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RELATIONSHIP OF THE PARATHYROIDS TO BONE PHOSPHORUS

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ABSTRACT

In an attempt to determine if parathyroid action on bone was directly related to the dissolution of bone crystals, rats were injected with radiostrontium and radiophosphorus at 18 hours and at 3 weeks prior to nephrectomy or nephrectomy-parathyroidectomy and peritoneal lavage. In the parathyroidectomized animals there was a decrease in both calcium and phosphorus removal during the lavage, the decrease in phosphorus being about half that of calcium. The amounts of radioactive isotopes removed by lavage were not affected by parathyroidectomy in the animals injected 18 hours prior to lavage. In the animals injected 3 weeks prior to lavage the amounts removed from the parathyroidectomized animals were less in all cases. Parathyroidectomy caused a 25% drop in radiostrontium removal and an 18% drop in radiophosphorus removal. The 2:1 decrease in weight of calcium over phosphorus removed and the 3:2 decrease in radiostrontium over radiophosphorus (radioactivity being based on atomic disintegrations rather than weight) after parathyroidectomy indicate strongly that the parathyroid hormone acts in bone at the level of previously formed bone crystal. It is suggested that the parathyroids maintain the calcium levels of the serum above a level which can be produced by a physicochemical equilibrium between bone surfaces and extracellular fluids.

FOR many years the most important consideration regarding the role of the parathyroids has been the ability of the hormone to produce an increased serum calcium level while concurrently lowering serum phosphate levels. In explanation of this phenomenon, two apparently opposing theories of parathyroid action arose, one postulating a bone effect (1) and the other postulating a renal effect (2). Eventually this led to consideration of a bone calcium-mobilizing action as compared to a phosphaturic action (3, 4). Reports from this laboratory have contended that the hormone in acting on bone must affect both calcium and phosphorus and in addition that *both* ions are likely affected by parathyroid influence on renal excretion (5, 6). It has been suggested that parathyroid hormone functions in bone

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through an ability to cause dissolution of previously formed bone crystal (7). However, observations have been limited for the most part to a demonstration of calcium dissolution. It is quite obvious that in the breakdown of bone crystal *per se*, both calcium and phosphate must be released in the ratio in which they occur in the crystal, which on an atom basis is three calcium atoms to two atoms of phosphorus, and by weight a ratio of 2:1.

Experiments using peritoneal lavage in rats (8) and continuous versene administration in dogs (9) have shown the ability of the parathyroids to mobilize large amounts of calcium from the bone. In the lavage experiments, parathyroidectomy caused a marked decrease in the total amount of calcium removed. However, the amount of tagged calcium or strontium removed from the bone was not decreased unless the radioactivity had been injected an adequate time before the experiment (10). These data were interpreted as indicating that the parathyroids removed calcium from the deeper areas of bone rather than the more readily available surface areas.

The present studies were undertaken to determine whether radiophosphorus was handled in a manner similar to radiocalcium, and to study the relationship of calcium and phosphorus removal from bone in an effort to determine if parathyroid action on bone was directly related to the dissolution of bone crystals.

MATERIALS AND METHODS

Male Holtzman rats, weighing between 190 and 230 gm., were used in these experiments. Peritoneal lavage was performed by the basic methods previously described (8, 11). In these experiments, parathyroidectomy was performed immediately prior to the lavage procedure. The lavage rinse was a calcium- and phosphate-free 0.84% NaCl solution containing 1.5% glucose. In previous work, 30 ml. of the rinsing solution were placed in the animal at the beginning of each successive wash and immediately removed. A second 30 ml. rinse was allowed to equilibrate intraperitoneally. The initial rinse was omitted in the present work because of the necessity to account for all the radioactivity removed.

Radiostrontium (Sr^{85}) and radiophosphorus (P^{32}) were administered simultaneously by intraperitoneal injection. Approximately half of the animals received a single injection containing 3 μc Sr^{85} and 3 μc of P^{32} six hours after nephrectomy. These animals were used in lavage experiments the following day. The remaining animals received five daily injections of the isotopes at the above mentioned level, the first injection being given three weeks prior to the lavage. The remainder of the experimental handling of these two groups was identical.

Measurements of Sr^{85} were made using a NaI scintillation crystal and pulse height analyzer. P^{32} measurements were made with a standard GM counter and scaler. Calcium determinations were made with a flame spectrophotometer by a procedure involving precipitation with ammonium oxalate and redissolution in percholoric acid. Total calcium, phosphorus and radioactivity measurements were made on the skeletons of all animals. These skeletons were defleshed and all soft tissues removed in a water medium by bacterial decomposition. Skeletons were then washed, dried to constant weight, and dissolved in nitric acid for the subsequent analyses. All the data were analyzed using the Student's T test for significance of differences between means.

RESULTS

The results presented below are divided into three groups. The first group is a comparison of the quantities of stable calcium and phosphate removed by peritoneal lavage in control and parathyroidectomized-nephrectomized rats. The second series deals with a comparison of the removal of the two different radioisotopes used under the same experimental conditions. The third group relates the amount of stable elements and radioactivity removed to amounts in bone, amounts administered, etc.

A. *Effect of parathyroidectomy on quantities of total calcium and phosphorus removed by continuous peritoneal lavage.*

These data are summarized in Table 1. The lavage removed 0.5 mg./hr.

TABLE 1. EFFECT OF PARATHYROIDECTOMY ON REMOVAL OF CALCIUM AND PHOSPHATE BY CONTINUOUS PERITONEAL LAVAGE[1]

	Expt'l. Condition	No. of Animals	Lavage Periods (60 Minute Washes) 1	2	3	4	5	6	7	8	9	10	11	12
Calcium Removed (mg./Lavage)	Nephx.[2]	11	1.53[3] ± .08	1.66 ± .05	1.60 ± .05	1.52 ± .07	1.50 ± .07	1.63 ± .05	1.72 ± .04	1.76 ± .06	1.74 ± .06	1.68 ± .08	1.79 ± .04	1.85 ± .04
	Ptx-Nephx.	11	1.10 ± .03	1.06 ± .03	1.09 ± .03	1.06 ± .04	1.09 ± .04	1.20 ± .05	1.28 ± .06	1.30 ± .05	1.34 ± .04	1.34 ± .05	1.33 ± .08	1.29 ± .06
Phosphate Removed (mg.P/Lavage)	Nephx.	11	2.5 ± .1	2.4 ± .06	2.3 ± .08	2.0 ± .09	2.0 ± .1	2.1 ± .09	2.2 ± .05	2.3 ± .1	2.1 ± .06	2.2 ± .09	2.1 ± .08	2.2 ± .05
	Ptx-Nephx.	11	2.3 ± .1	2.1 ± .1	2.0 ± .1	1.8 ± .09	1.9 ± .1	1.9 ± .09	2.0 ± .09	2.0 ± .08	2.1 ± .07	2.0 ± .09	1.8 ± .1	2.0 ± .09

[1] For ease of comparison of results, the average radioactivity—removed per lavage period in the control group in any one experiment was set at a 100 counts per minute and all values for that experiment adjusted accordingly.
[2] Nephx = Nephrectomized only; Ptx-Nephx = Parathyroidectomized and Nephrectomized.
[3] Mean ± standard error $\sqrt{\frac{\Sigma d^2}{n(n-1)}}$

less calcium in the parathyroidectomized than in the control animals. For phosphate, this decrease was 0.25 mg.P/hr. or about one-half that of calcium. On a weight basis, more phosphorus (as phosphate) than calcium was removed per hour in both groups. Since phosphate values were more variable than calcium values and the differences between the two groups less, the statistical significance for phosphate changes at any one hour was borderline. However, based on differences in successive groups, it could be shown that the differences between the two groups were significant at the 1% level.

B. *The effect of parathyroidectomy on removal of radiostrontium and radiophosphorus.*

Radiostrontium was used instead of radiocalcium because its emission energies allowed separate determinations of Sr^{85} and P^{32} content of the same sample. Earlier studies (11) have shown that there was a slight preferential removal of strontium over calcium from bone, but that basically it was handled similarly to calcium. Changes in radiostrontium removal in this experiment are therefore considered to be indicative of radiocalcium changes.

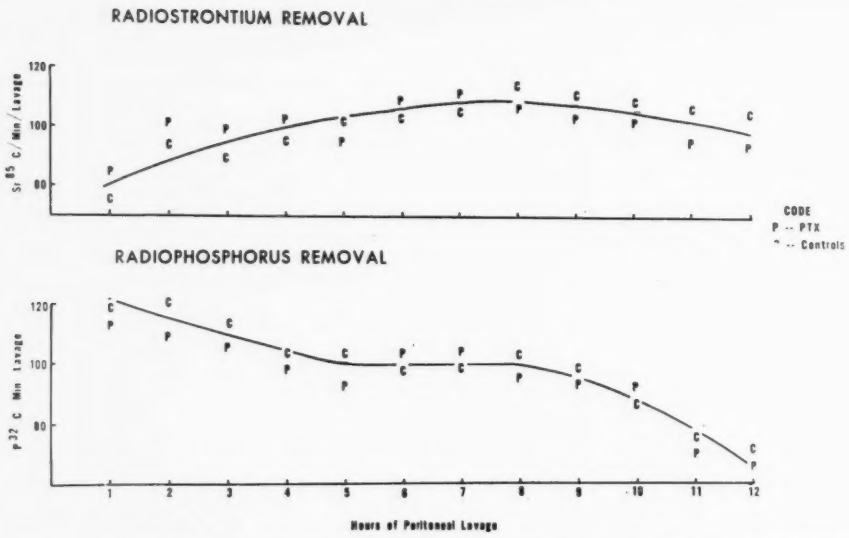

FIG. 1. Removal of P^{32} and Sr^{85} by peritoneal lavage: radioisotopes injected 18 hours prior to lavage. Animals parathyroidectomized prior to first lavage period.

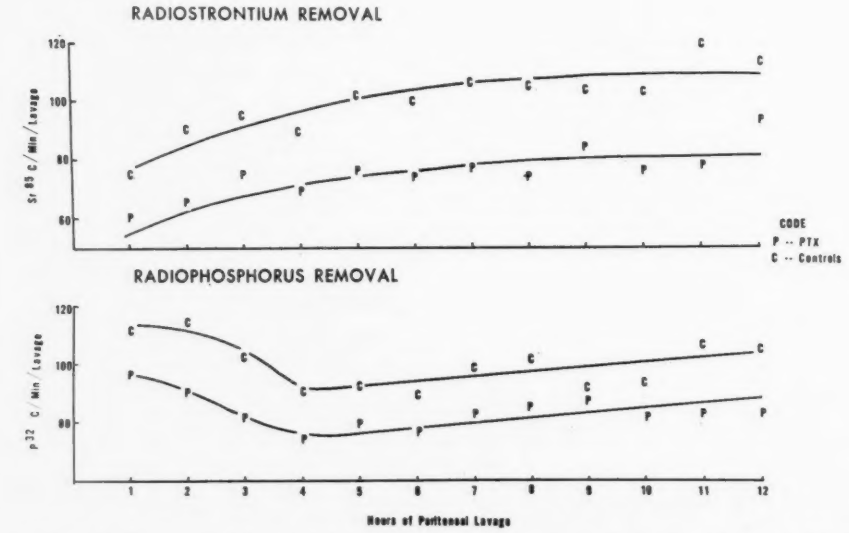

FIG. 2. Removal of P^{32} and Sr^{85} by peritoneal lavage: radioisotopes injected third week prior to lavage. Animals parathyroidectomized prior to first lavage period. Based on total radioactivity removed during 12 hours of lavage, parathyroidectomy produced a 25% drop in Sr^{85} removed (statistical significance of drop: $P < 0.1$); and an 18% drop in P^{32} removed (statistical significance of drop: $P = 0.8$).

The data are summarized in Figures 1 and 2. The amounts of radioactivity removed by lavage were not affected by parathyroidectomy if the activity was injected within 24 hours of the start of the lavage. However, if the activity was administered to the animals three weeks prior to use, the amounts removed by lavage from parathyroidectomized animals fell in all cases. Parathyroidectomy produced a 25% drop in radiostrontium removal while the radiophosphorus drop was 18% or approximately two-thirds that of the radiostrontium.

C. *Relative removal of calcium, radiostrontium, stable and radioactive phosphorus.*

In Table 2 are shown the percent of the injected dose removed by lavage,

TABLE 2. RELATIVE REMOVAL OF RADIOSTRONTIUM AND RADIOPHOSPHORUS BY PERITONEAL LAVAGE

		Exptl. Condition	No. of Animals	% Inj. Dose Removed	Skeletal Dose (% Inj. Dose in Skel.)	% Skel. Dose Removed	Sp. Act.[3] (Rel.) Act.) in wash cts/min/ml	Sp. Act.[3] (Rel. Act.) of Skeleton cts/min/mg.
Group[1] A	Sr^{85}	Nephx[2]	6	6.6±0.2[4]	82.6±3.5	8.0±0.3	2534±110	302±24
		Ptx-Nephx	6	6.6±.04	88.6±1.1	7.6±0.5	3670±275	319±18
	P^{32}	Nephx	6	9.9±0.3	51.7±1.7	18.9±0.7	10940±266	1368±48
		Ptx-Nephx	6	9.7±0.5	55.6±1.1	17.5±0.9	12242±586	1524±56
Group[1] B	Sr^{85}	Nephx	5	0.5±0.02	51.8±1.4	0.9±0.04	973±33	1004±27
		Ptx-Nephx	5	0.4±0.01	49.1±1.7	0.7±0.07	985±85	936±26
	P^{32}	Nephx	5	1.3±0.05	43.6±0.7	3.0±0.1	3565±87	3338±98
		Ptx-Nephx	5	1.1±0.04	41.8±0.6	2.7±0.1	3251±139	2948±31

[1] Group A received injection dose of 8.05×10^6 cts/min. Sr^{85} and 3.1×10^6 cts/min. P^{32} 24 hours prior to lavage. Group B received injection dose of 3.88×10^6 cts/min. Sr^{85} and 6.68×10^6 cts/min. P^{32} 3 weeks prior to lavage.

[2] Nephx = nephrectomized only; Ptx-Nephx = parathyroidectomized-nephrectomized.

[3] Specific activity is defined as cts/min/mg of stable isotope. For Sr^{85} figures represent relative activity (cts/min Sr^{85}/mg. Ca^{40}).

[4] Mean ± standard error $\sqrt{\frac{\Sigma d^2}{n(n-1)}}$.

the percent of the skeletal dose involved, and changes in specific activities of the radioisotopes injected. Pertinent to the discussion are the facts that far larger percentages of the injected doses of radiophosphorus were removed than those of radiostrontium and secondly that the specific or relative activities were affected to a major extent only in those experiments where the radioactivity was injected within the twenty-four hours preceding the lavage.

DISCUSSION

Previous work (10) has suggested that calcium is removed from bone by two processes. The first is basically a physicochemical equilibrium between extracellular fluid and those portions of bone readily accessible to the fluid. The second process is considered to be actual bone dissolution occurring in the deeper trabecular areas of bone. The rate of this process is believed to be influenced by the parathyroid hormone. If this hormone is actually concerned with the dissolution of bone crystal, then it follows that calcium and phosphate liberated by hormone action should be in the proportions that they are considered to exist in the crystal. The data in Table 1 show

that parathyroidectomy causes a decrease in calcium removal which by weight is twice as large as the decrease in phosphorus removal. In addition the radioactivity data (Figure 2) which are based on the numbers of atoms concerned regardless of weigh⁺, show a removal ratio of strontium to phosphorus of 3:2. The combined interpretation of these two groups of data indicate very strongly that parathyroid hormone acts in bone at the level of previously formed bone crystal.

The suggestion that there is a major process of calcium removal from bone with which the parathyroids are not concerned has been derived from the fact that radiocalcium or radiostrontium recently incorporared into bone cannot be influenced by parathyroidectomy or citrate administration (10). These earlier data are further borne out in this study wirh radiophosphorus. Recently deposited radioactivity (Fig. 1), radiophosphorus and radiostrontium, is not affected by parathyroidectomy despite the drop in total calcium and total phosphate removed. One can conclude that the function of the parathyroids is to maintain the calcium level of the body fluids above the level which can be produced by the physicochemical equilibrium between bone surfaces and extracellular fluids.

One additional problem raised by these studies is the apparent removal of almost 1.5 times as much skeletal phosphorus as calcium during the lavage periods based both on calculations of quantities of stable elements and by radioactivity determinations. The data indicate that this is a problem not connected with the function of the parathyroid hormone since parathyroidectomy does not affect this removal ratio significantly. While no explanation of the differential removal of these two ions can be given, it is suggested that it could be the result of the relative difference in size of the available phosphate and calcium pools in extraosseous areas.

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EXTRACTION OF THYROTROPHIN FROM PITUITARY GLANDS, MOUSE PITUITARY TUMORS, AND BLOOD PLASMA BY PERCOLATION

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ABSTRACT

A percolation method of extraction of thyrotrophin from dry powders of anterior pituitary glands, mouse pituitary tumors and blood plasma has been developed. The powder mixed with an equal weight of filter aid suspended in 95% ethanol was placed on a Buchner funnel and increasingly more aqueous solutions containing salt were run in succession through the filter cake in a stepwise manner. The TSH is removed with 57 or 67% ethanol solutions saturated with sodium chloride. The yields were estimated to be over 50% of the potency in about 2% of the weight, which is a concentration of the activity of about 30 times, whether from dried plasma or pituitary powder.

THE six known hormones of the anterior pituitary gland have been found to be proteins of relatively low molecular weight (<50,000) and are more stable during solvent fractionation than many proteins (1).

Most of the published extraction procedures have used aqueous solvents which remove much hormonally inactive protein along with the active hormones. Subsequent steps have included fractionation by slow addition of some salt, acetone, or ethanol with appropriate pH adjustment.

Since most of the anterior pituitary hormones tend to be more soluble than the associated inactive contaminants, the conventional process was reversed by extracting the more soluble pituitary hormones first. Thyrotrophin (TSH) has been successfully extracted by a column type percolation technique starting with acetone dried or lyophilized glands suspended in 95% ethanol and proceeding in a stepwise manner toward an aqueous medium. When applied to blood plasma, the TSH was extracted in high yield with only 2 to 5% of the total solids of dried plasma.

METHODS AND MATERIALS

An acetone dried, bovine anterior pituitary powder #12830[1] was used in most of these experimental studies. Sheep anterior pituitary powder #21757 was a lyophilized product. Powder from transplantable, thyrotrophin-producing, mouse pituitary tumor, Strain

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[1] Obtained through the courtesy of the Endocrine Study Section, National Institutes of Health.

77D, was obtained by lyophilization. Lyophilized human plasma which was several years old was non-sterile Red Cross plasma. All powders were run through a 40 or 60 mesh screen in a Wiley mill. NaCl–CP and 95% ethanol, U.S.P., were used. Hyflo-Super Cel (Johns Manville) was used as a filter aid.

TSH potency was determined by the I^{131} depletion method of Bates and Cornfield (2) and the values found are expressed as U.S.P. units. Assays for TSH activity on the individual tubes of the columns were ±50%, and on the pooled fractions were ±30%. Prolactin was determined by the pigeon cropsac weight method of Bates and Riddle (3). Only 2 pigeons were used for each tube, hence values are only approximate.

Percolation was carried out on Buchner funnels usually having a sintered glass plate and a precoat of Hyflo. A 500 cc. suction flask graduated at 50 and 100 cc. or with a small graduated tube inserted was used to collect the percolates. For ten gram samples, a 45 mm. diameter funnel was used and 10 cc. fractions were collected in a graduated test tube. For 100 gm. samples a 93 mm. diameter funnel was used and 50 cc. fractions were collected. Height of the cake on the funnel varied with batch size from 40 to 70 mm. The flow was increased and regulated by weak or intermittent suction, so that the collection of each fraction took 5 to 10 minutes. A total of 30–40 fractions could be collected in a day. The specific gravity of the percolates was determined by weighing or with small hydrometers. Absorbancy at 276 mμ was determined in a Beckman Model DU spectrophotometer. All experiments were carried out at room temperature.

Two of the problems which had to be faced in the percolation procedure were the maintenance of an adequate flow rate and the prevention of shrinkage of the column from the wall of the funnel. It was found that the addition of a half to an equal part by weight of Hyflo to a powder solved the flow rate problem and modified the shrinkage. A proper sequence of solvent mixtures had to be chosen to avoid shrinkage at any stage.

Description of a typical percolation

The procedure described is specifically that used for the run presented in Figure 3. 100 gm. of bovine acetone dried pituitary powder #12830 was mixed dry with 50 gm. of Hyflo and suspended in 400 cc. of 95% ethanol. This slurry was poured in 100 cc. portions onto a 93 mm. Buchner funnel, which had been precoated with Hyflo. Each portion was allowed to drain free of supernatant ethanol before the next portion was applied. The first 50 cc. or so of filtrate was used to transfer the last of the slurry onto the funnel. From the volume of the percolate (120 cc.), when the free liquid had just disappeared from the top of the cake, the retention volume of the cake was found to be 280 cc. (400 cc. − 120 cc.).

Five hundred cc. of a solution made up of 10 gm. of NaCl in 100 cc. H_2O to which has been added 400 cc. of 95% ethanol, were then poured on top of the column. This solution was approximately 76% ethanol by volume and contained 2% NaCl. When 280 ml. more of percolate was accumulated, completing the collection of the 400 cc. of 95% ethanol, the collection of samples of 50 cc. volume was begun. The specific gravity and absorbancy of the whole 95% ethanol percolate is indicated as the "O" tube in most of the figures, and the volume of this "O" sample would be that of the amount of 95% ethanol needed to pour the column (400 cc. in this case).

When the 500 cc. sample of 76% ethanol − 2% NaCl had all run into the cake, the next more aqueous solvent 67% ethanol − 5% NaCl, was poured on top, and so on in this manner to the end of the run (as in Fig. 3). An excess (280 cc. in this case) of the last solvent was needed to complete the run to tube 34. Thus, there was always a lag of about 280 cc. of percolate between the placing of a solvent on top of the cake and its appearance from the bottom. This large holdup volume was the reason for determining the specific gravity of the percolates. Where solvent fronts were observed, they were

surprisingly horizontal and the breaks in the specific gravity curves bore this out. Percolates were pooled and concentrated to a saturated NaCl solution in a rotary evaporator with the condenser cooled with dry ice. The saline concentrates were then dialyzed overnight in cellophane tubing against running tap water. Isoelectric precipitates were removed from the dialyzed solutions after the addition of dilute H_2SO_4 to approximately pH 4.5. The separated pH 4.5 soluble and insoluble fractions were adjusted to pH 8–9 and lyophilized.

RESULTS

Percolation with aqueous ethanol

When progressively more aqueous solutions of ethanol were percolated through acetone dried bovine pituitary powder, no TSH appeared in the percolate until the concentration of ethanol was considerably less than 50% (Fig. 1).

Effect of NaCl on extraction of TSH with 57% ethanol

Another percolation (Fig. 2) was carried out with stepwise increases of water concentration through the 57% ethanol step. No TSH appeared, confirming the data in Figure 1. Then a solution of 57% ethanol containing 8% NaCl was passed through the column. With the emergence of the salt solution at tube 14 there was a sharp peak in the protein concentration, and with this peak appeared most of the TSH (Fig. 2). NaCl is thus seen

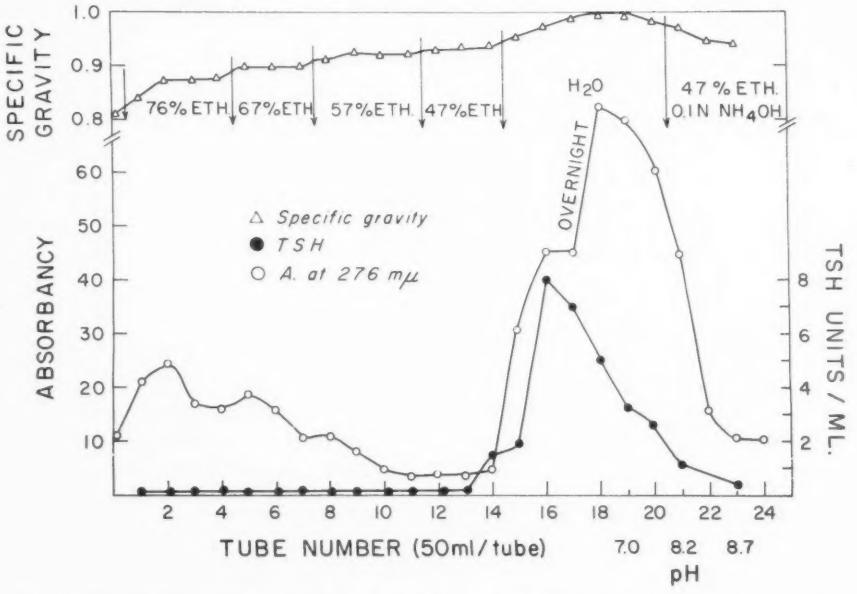

FIG. 1. Percolation without added salt. 70 gm. acetone-dried bovine pituitary powder mixed with 50 gm. Hyflo in a 93 mm. diameter funnel. Column height = 45 mm.

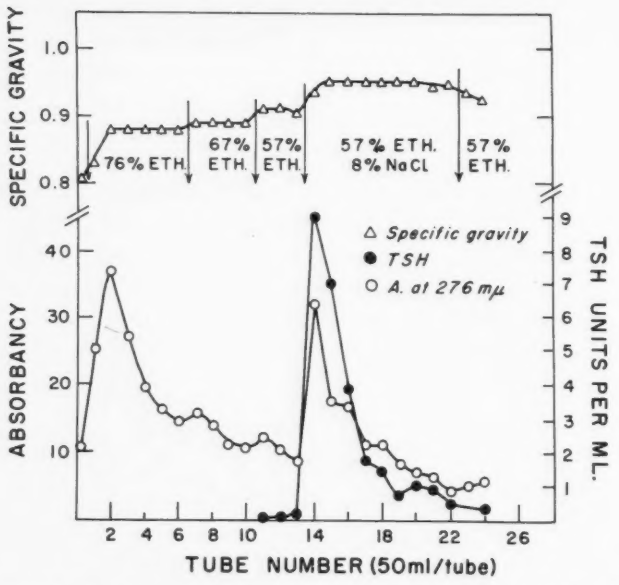

FIG. 2. Percolation with salt added following percolation without salt. 100 gm. acetone-dried bovine pituitary powder mixed with 50 gm. Hyflo in a 93 cm. diameter funnel. Column height = 50 mm.

to increase the solubility in aqueous ethanol of TSH and certain other proteins.

Percolation using NaCl solutions

The concentrations of NaCl used in this experiment were those needed to saturate the solution, except when the ethanol concentration was less than 50% (Fig. 3). No TSH was extracted by the 76% ethanol solvent containing 2% NaCl. When the ethanol concentration fell to 67% at tube 10, there was a small increase in the protein concentration, as measured by the absorbancy at 276 mμ, and a sharp increase in the solubility of TSH. When the ethanol concentration was decreased to 57% at tube 18, more TSH was extracted without an appreciable alteration in protein concentration. The higher ratio of TSH to absorbance at 276 mμ indicated a higher specific activity in fractions 18 to 21. The lyophilized isoelectrically soluble protein fraction from the pooled 57% ethanol–7% NaCl percolates regularly have TSH potencies of 1 to 2 units/mg. (Table 1). This is a very high potency for such a simple fractionation. Similar 67% ethanol-saline fractions have TSH potencies of 0.5–1.0 units/mg. No additional proof that the TSH in these fractions differ has been found.

This percolation sequence has been repeated ten times with slight modifications, but with similar results. This was the procedure chosen for study of blood plasma.

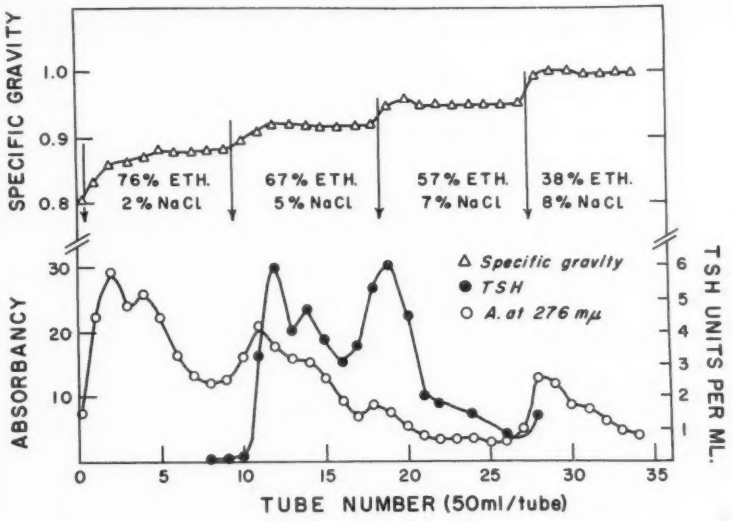

FIG. 3. Percolation with solutions nearly saturated with salt. 100 gm. acetone-dried bovine pituitary powder mixed with 50 gm. Hyflo in a 93 mm. diameter funnel. Column height = 50 mm.

Extraction of prolactin by percolation

Lyophilized sheep anterior pituitary powder, #21757, was extracted by percolation in order to get an estimate of where sheep TSH and prolactin would be found in the procedure. The absorbance of the percolates (Fig. 4) was very similar to that found with bovine glands (Fig. 3). Unlike the usual finding with bovine glands, the first solubility peak of TSH activity was lower than the second peak, but this might be due to instability. By direct assay of the percolates, most of the prolactin was found in the 76% ethanol–2% NaCl percolate before the TSH appeared, but it trailed

TABLE 1. DISTRIBUTION OF WEIGHT AND POTENCY OF TSH IN PROTEIN FRACTIONS OBTAINED BY POOLING PERCOLATES USING THREE DIFFERENT STARTING POWDERS

Percolating solution	Bovine Anterior Pituitary 300 gm.–0.035 U/mg.				Mouse Tumor—77D 10 gm.–0.1 U/mg.				Human Plasma 80 gm.–900 ml.		
	pH 4.5 soluble			pH 4.5 insol.	pH 4.5 soluble			pH 4.5 insol.			
	% wt.	U/mg.	% U	% wt.	% wt.	U/mg.	% U	% wt.	% wt.	mU/mg.	*% U
76% ethanol 2% NaCl	0.1	0.025	0.1	0.2	0.6	0.015	0.9	1.7	0.23	0.2	8.9
67% ethanol 5% NaCl	1.2	0.8	28	1.1	0.64	1.5	9.6	0.24	0.85	0.13	20.0
57% ethanol 7% NaCl	0.8	2.0	48	0.6	0.88	3.4	30.0	0.46	0.77	0.4	55.6
38% ethanol 8% NaCl	—	—	—	—	—	—	—	—	0.54	0.15	15.5
TOTAL	2.1		76	1.9	2.12		40.5	2.40	2.39		100.0

* %based on recovery.

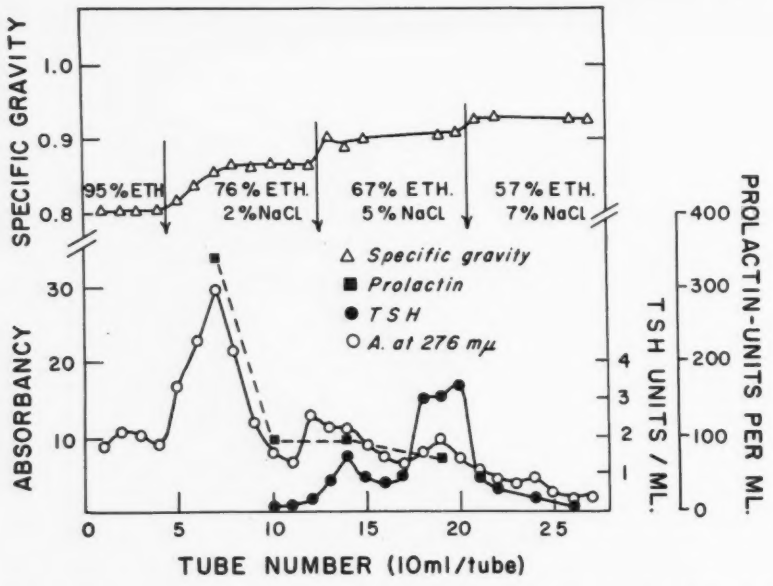

FIG. 4. Extraction of TSH and Prolactin by percolation. Ten gm. lyophilized sheep pituitary powder mixed with 10 gm. Hyflo in a 45 mm. diameter funnel. Column height = 37 mm.

throughout the TSH fractions. The yield of prolactin based on admittedly inadequate assays was in the theoretical range.

Percolation of mouse pituitary TSH tumor powder

When transplantable mouse pituitary TSH tumor powder from Strain 77D was extracted by percolation (Fig. 5) a pattern similar to that of bovine pituitary powder (Fig. 3) was obtained. Minor differences of possible significance were the high absorbance peak with the 67% ethanol solution and the relatively lower TSH concentration at that point.

Since the TSH content of the tumor powder was 3 times that in the bovine pituitary powder (4), the TSH concentration per cc. of percolate was also 2 to 3 times greater. The lyophilized isoelectrically soluble protein fraction from tubes 23–28 also had a correspondingly high potency of 3.4 U/mg. (Table 1).

Percolation of human plasma

Lyophilized human plasma was milled through a 40 mesh screen, then mixed with an equal weight of Hyflo and percolated using the usual sequence of solvents (Fig. 6). The protein elution pattern was somewhat similar to that obtained with pituitary and tumor tissue, but the solubility as measured by the absorbance was one-third to one-fifth of that obtained

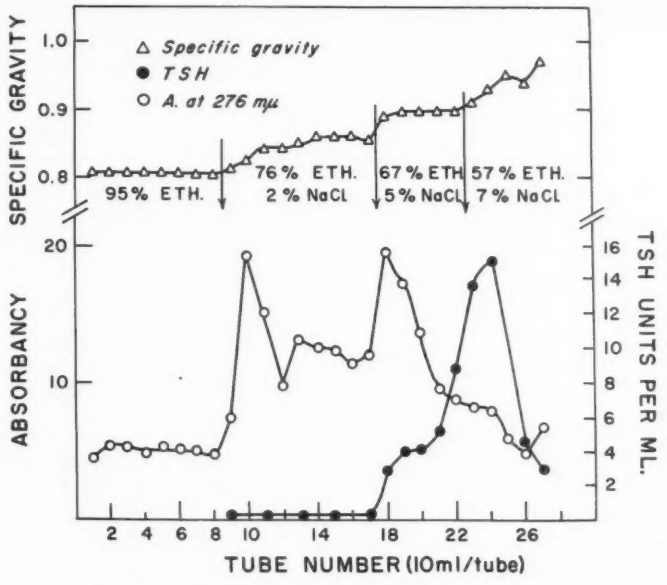

FIG. 5. Percolation of transplantable mouse pituitary tumor Strain 77D. Ten grams mixed with 10 gm. Hyflo in 43 mm. diameter funnel. Column height = 40 mm.

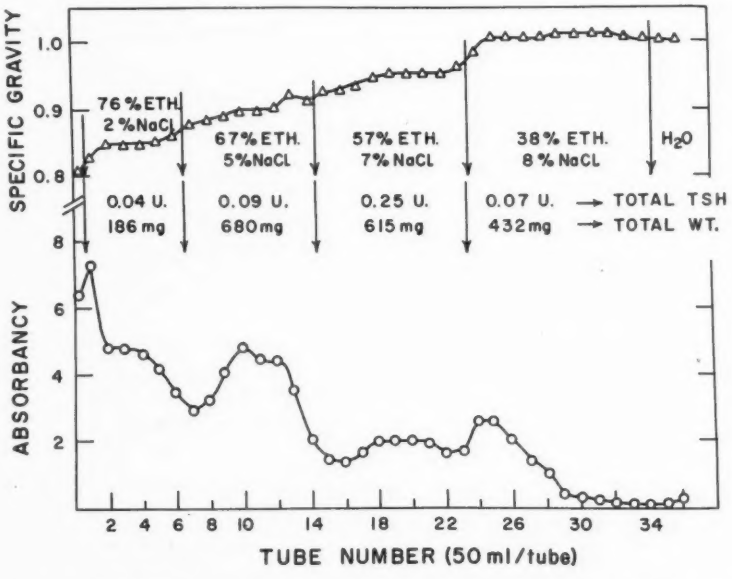

FIG. 6. Percolation of human plasma. 80 gm. lyophilized human plasma milled to 40 mesh (from about 900 ml. plasma) mixed with 80 gm. Hyflo in a 93 mm. diameter funnel. Column height = 70 mm.

with an equal weight of the glandular tissues. The extent of denaturation of the bulk of the plasma proteins is shown by the low solubility of the proteins in water in tubes 35 and 36. This denaturation probably contributes to the success of the percolation procedure in case of glandular tissue as well as plasma.

The content of TSH was too low to measure on the percolates directly. Hence, the pooled fractions were not assayed until after concentration, dialysis, and lyophilization (Table 1). The TSH potency per mg. of the main fraction isolated (0.4 mU/mg.) is one five-thousandth that of the main fraction from bovine pituitaries. The total TSH potency was 0.45 U from approximately 900 cc. of plasma, or a yield of 0.5 mU/cc. of plasma.

The 67% and 57% ethanol-saline fractions from another large percolation run with human plasma were pooled to make enough material for a 3 dose level bioassay (Fig. 7). Nearly identical values for I^{131} depletion were obtained during each 24 hour period at each dose level and these also agreed with the 48 hour depletion values. The slope of the dose response curve doesn't differ from that found with pituitary TSH. This suggests that the fractions from plasma truly contain TSH.

Percolation of plasma from mice with TSH tumors

Plasma from nearly 200 mice with large TSH-producing transplantable pituitary tumors of 4 different strains were frozen, lyophilized and then

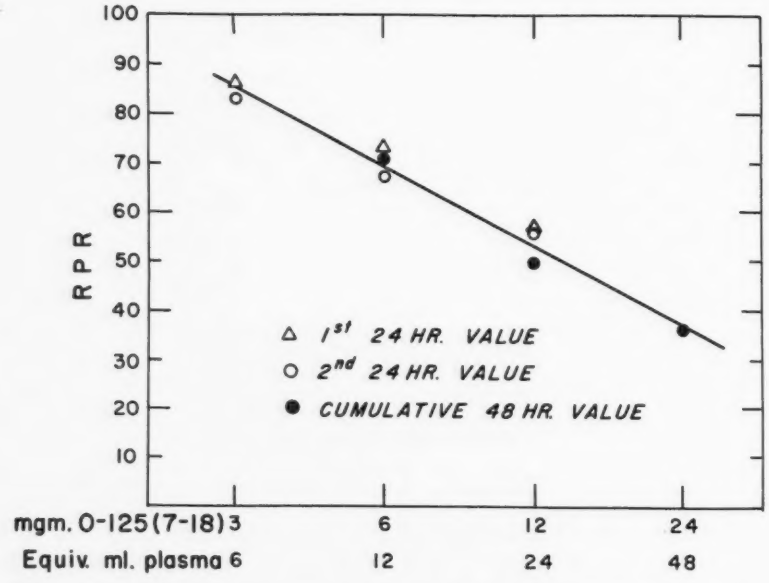

FIG. 7. Dose-response curve for bioassay of TSH from human plasma using I^{131} depletion in the baby chick. Ordinate "R.P.R." is the relative per cent of I^{131} remaining in the thyroids after the three time periods shown.

TABLE 2. COMPARISONS OF THE TSH CONCENTRATION OF NORMAL HUMAN BLOOD PLASMA AND OF BLOOD PLASMA AND TUMOR TISSUE FROM MICE WITH LARGE TRANSPLANTABLE PITUITARY TUMORS. THE POTENCY OF THE POOLED PERCOLATES AND THE EXTENT OF PURIFICATION ARE ALSO SHOWN

Tissue	Fresh tissue mU/cc. or gm.	Dried tissue mU/mg.	Combined 57%+67% fractions mU/mg.	Purification
Normal human plasma	0.5–1.0*	0.005–0.01*	0.3	30–60×
Tumor mouse plasma	1,000.	10.	300.0	30×
Tumor tissue	20,000.	100.	3,000.	30×

*Estimated from potency of extracts.

pooled. Six grams were available for percolation. Such plasma usually has a potency of about 1 U/cc. so that only 0.005 cc./chick is sufficient for assay purposes and the potency estimates on the unfractionated plasma are reliable (Table 2). The plasma powder, after lyophilization, had a TSH potency of 0.01 U/mg., which is one-third that of dried anterior pituitaries of cattle or sheep and one-tenth that of dried mouse and rat pituitaries. After percolation of the dried plasma in the usual manner, the 67% and 57% ethanol-saline percolates were pooled, dialyzed and lyophilized. An 80 mg. fraction was obtained that had a potency of about 0.3 U/mg. and represented a recovery of some 40% of the activity in 1.3% of starting solids by weight. This 40% recovery of TSH activity agrees with the yield found with pituitary and tumor tissue (Table 1). Hence, the true value for TSH content of pooled human plasma might be twice 0.5 mU/cc. or 1 mU/cc.

Table 2 shows the 1,000 to 2,000 times greater concentration of TSH in the plasma from TSH tumor mice than in normal human plasma and also shows that the percolation procedure concentrates this activity in 1% to 2% of the plasma solids with an overall recovery of slightly less than 50% of the TSH. This results in an increase of 30 to 50 times in the TSH activity per mg. of protein, regardless of the potency of the starting material. The data of Table 2 supplement the data of Table 1, which show that the pH 4.5 soluble 67% and 57% ethanol-saline fractions represent 1% to 2% of the starting solids, whether plasma or glandular in origin, and contains 40–70% of the TSH activity. As expected, the pH 4.5 insoluble fractions are not present in plasma extracts.

Yield of TSH from human plasma

When unfractionated human plasma from normal individuals was injected into chicks, a significant I^{131} depletion was found with only a few plasmas and then only after 6 cc. of plasma/chick/day was injected. This would indicate a TSH content of about 0.3 mU/cc. of plasma. A dose of 6 cc./day sucutaneously in a 40 gm. chick would seem to be unphysiclogical and larger doses were not tried. As shown in Figure 7, 3 mg. of extract (equivalent to 6 cc. of plasma) gives, after 24 hours, an RPR of 85%,

which was significantly different from the RPR of 95% in control chicks. The 30% depletion (RPR-70%) with 6 mg. of extract (12 cc. plasma), whether in one injection or 2 daily injections of 3 mg., is greatly different from that of the control chicks. Reckoned against the crude assays on whole plasma, these data indicate a yield of TSH of more than 100% upon extraction from plasma. This unreal yield is probably due to the large volumes and high protein concentration of plasma, which adversely affect the rate of absorption of the TSH. To check this last point, TSH was added to plasma and injected in volumes of 3 cc. to 6 cc./day. Depletions indicated only 50% of the potency added. Hence, it is not possible at present in the baby chick to determine accurately the actual TSH potency of unfractionated whole plasma from normal individuals.

DISCUSSION

The data in Tables 1 and 2 show that the potency of the fractions obtained depends on the potency of the starting tissue, hence, extraction of TSH in the percolation procedure is not simply a matter of solubility of TSH. For example, the TSH concentration of bovine pituitaries is several thousand times that of human plasma (Table 1). The 76% ethanol-saline fractions from bovine glands and from human plasma have about the same yield of weight (0.1% vs. 0.23%), but differ by 89 times (0.1% vs. 8.9%) in yield of TSH potency. This is in the direction expected by simple solubility. But the greater activity in the 76% fraction in the case of plasma is still less than 10% of the total TSH activity which trailed even into the 38% ethanol fraction. The fact that only about a 30 to 50 times concentration of activity is obtained from either blood plasma or pituitaries would indicate an association of the TSH with proteins of similar solubilities and proportions in both cases or of binding with insoluble proteins in similar manner.

At the limited stage of purification obtained by percolation, no difference has been found in the TSH from various tissues, whether glandular or blood plasma, from man, ox, sheep, and mouse.

The effect of altering the pH of the tissue during percolation has been examined in a preliminary manner. By adding small amounts of HCl, acetic acid, or $NaHCO_3$ to the 95% ethanol slurry, percolations were carried out in the range of pH 5 to 8.5. No marked effect was found on the extraction of TSH, but there was some evidence that the TSH in the percolates was more stable at pH 8–8.5, when allowed to stand 24–48 hours at room temperature. Pierce (5) has recently shown that TSH passes through certain stretched cellophane membranes. No losses were encountered in the dialysates with the cellophane used in these experiments.

The concentration of TSH found in human blood plasma by the percolation procedure is consistent with that reported by Gilliland and Strudwick (6), using a similar bioassay procedure in baby chicks, by D'Angelo (7, 8),

using the increase in cell height in the thyroids of tadpoles, and by Adams (9), using the increase in level of I^{131} in the plasma of thyroxine treated guinea pigs.

Whether the extraction of TSH in the presence of Hyflo is dependent simply on solubility or whether chromatography is also a factor is a question that can be answered only partially. One attempt to answer this question was made by percolating bovine pituitary powder without Hyflo. Flow rate was good in 95% ethanol. Soon after the 76% ethanol–2% saline solution emerged in the percolate and before the next more aqueous solution emerged, the flow rate decreased rapidly and completely stopped, even under full vacuum. There was no TSH in the percolate up to the time of stopping. Since, in the presence of Hyflo, TSH would be expected in the next solvent change, this is evidence that the Hy-flo does not retard the elution of the TSH and serves mainly to increase the porosity of the tissue mass under the conditions of pH and salt concentration used. Preliminary runs with column heights of 23 cm. resulted in flow rates, even under full vacuum, of one-fifth those normally found. As a result, more of the TSH was in the 67% ethanol-saline percolate, but the concentration of TSH per cc. of percolate was not greatly increased. Thus, little is to be gained by thicker cakes or slower rates of flow. The available evidence indicates that chromatography is of minor or no significance in this percolation procedure.

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ALDOSTERONE SECRETION IN DOGS WITH HYPOTHALAMIC LESIONS[1,2]

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ABSTRACT

Aldosterone and 17-hydroxycorticoid secretion have been measured by collecting the effluent blood from the right adrenal in 6 dogs with lesions of the anterior median eminence, and 6 dogs with lesions elsewhere in the hypothalamus. These values have been compared to those obtained in 6 normal and 4 hypophysectomized dogs. Sodium and potassium levels in serum and urine have also been measured on a normal and a low sodium intake. Lesions of the anterior median eminence which inhibit hydroxycorticoid secretion have no effect on sodium and potassium metabolism, but are associated with a reduced aldosterone secretion in the adrenal venous blood after acute cannulation of the adrenal. Similar results, i.e., moderate impairment of aldosterone secretion in the absence of any detectable abnormality of sodium and potassium metabolism, were obtained in the hypophysectomized dogs. It is suggested that the observed differences between normal dogs and dogs with lesions of the median eminence are due to decreased ACTH secretion in response to the trauma of adrenal cannulation, rather than any direct effect on the mechanism regulating aldosterone secretion in response to changes in sodium and potassium intake.

DESPITE intensive study, the mechanism regulating aldosterone secretion remains an enigma. Farrell and his coworkers have suggested that the diencephalon is part of the regulatory mechanism. They base this conclusion on the observation that decerebration in the dog (1), and acute lesions of the ventral hypothalamus in the cat (2) depress the secretion of aldosterone. In dogs with chronic ventral hypothalamic lesions and a decreased 17-hydroxycorticoid response to trauma, Daily and Ganong (3) have reported that urinary and serum levels of sodium and potassium are nor-

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mal not only on a normal sodium intake, but when the animals are placed on a low sodium, high potassium diet. Since adrenalectomized dogs treated with glucocorticoids, but no mineralocorticoids, became hyponatremic and died on the same regime, it seemed likely that no appreciable adlosterone deficiency was present in the dogs with lesions. It seemed important, however, to measure aldosterone secretion directly in a group of animals with hypothalamic lesions in which sodium and potassium metabolism had been studied. Accordingly, the following experiments were carried out.

MATERIALS AND METHODS

Twenty-two male mongrel dogs weighing 8.0 to 22.2 kg. were studied. Electrolytic lesions were produced stereotaxically by the technique of Hume and Ganong (4) in the anterior median eminence of six of these dogs (Group 2) and in other portions of the hypothalamus in another six animals (Group 3). Four dogs were hypophysectomized by the transbuccal route, and six normal animals used as controls (Group 1). All operated animals were permitted to recover for three weeks before studies were begun.

Urinary excretion of sodium and potassium and serum sodium and potassium levels were measured in the dogs with hypothalamic lesions on a diet containing 40 m.eq. of sodium and 8 m.eq. of potassium for two days, and on a diet containing 0.5 m.eq. of sodium and 50 m.eq. of potassium for three days. Two of the four hypophysectomized dogs were similarly studied. The technique used in these metabolic studies has been described in detail elsewhere (3). The data thus obtained were compared to that obtained in 15 normal animals. Results in the normal and hypophysectomized animals have been previously published (3).

After the above studies were completed, the animals were maintained an additional two days on the low sodium regime, and then subjected to right adrenal vein cannulation by the technique of Hume and Nelson (5) under pentothal-induced ether anesthesia. Plastic cannulae were also placed in the femoral artery and femoral vein and the blood pressure was continuously monitored with a mercury manometer. One to three-minute adrenal venous samples were obtained for 17-hydroxycorticoid determination. The dogs were then bled from the femoral artery into a plastic blood bag (Fenwal Laboratories Inc.) an amount of blood equal to 20 ml./kg. of body weight. A two-hour adrenal venous blood collection was started immediately after completion of the bleeding. In an effort to keep the blood volume as constant as possible during this two-hour period, the dogs were transfused from the blood bag through the femoral vein catheter every five minutes with an amount of blood equal to their adrenal venous blood flow.

Adrenal venous samples were collected in iced tubes, centrifuged, and the plasma promptly frozen. At the end of the procedure, the animals were killed. In the animals with hypothalamic lesions and the hypophysectomized dogs, the hypothalamus and the pituitary region were dissected out in one piece, fixed in 10% formalin and subsequently serially sectioned at 16μ. The sections were stained with hematoxylin and eosin and used to reconstruct the lesions or check the completeness of hypophysectomy. All hypophysectomies were found to be complete. The adrenals and testes were also cleaned, weighed and fixed in formalin. Representative sections of each of these organs and the kidney were stained with hematoxylin and eosin and examined.

17-hydroxycorticoid determinations were carried out by the method of Silber and Porter (6). Aldosterone was extracted from plasma with chloroform. The extracts were washed with 0.1 volume of cold 0.1N sodium hydroxide and water, dried over anhydrous sodium sulfate, and evaporated to dryness at 40° C. After chromatography in a chloroform-formamide system, the zones corresponding to the cortisone standard were eluted

TABLE 1. ALDOSTERONE BIOASSAY RESULTS. VALUES ARE DOCA EQUIVALENTS ±95% CONFIDENCE LIMITS OF EACH ASSAY. THE FIGURES IN PARENTHESES ARE THE FRACTION OF THE TOTAL 2-HOUR ADRENAL VENOUS PLASMA FLOW USED IN EACH ASSAY.

Normal Dogs	Dogs with lesions		Hypophysectomized dogs
	Involving anterior median eminence	Sparing anterior median eminence	
8.8±2.2 (.80)	4.6±2.3 (1.0)	8.1±2.2 (.80)	0±2.3 (.80)
6.2±2.2 (.80)	1.4±2.3 (.80)	5.6±2.3 (.67)	5.0±2.2 (.80)
5.3±2.3 (.67)	1.7±2.2 (.80)	5.3±2.3 (.80)	1.7±2.3 (.80)
6.1±2.2 (.67)	10.3±2.7 (1.0)	5.7±2.2 (.80)	0±2.3 (.80)
7.5±2.2 (.33)	3.5±2.2 (.80)	9.2±2.3 (.67)	
2.3±2.2 (.67)	4.1±2.3 (.80)	7.2±2.2 (.40)	

and bioassayed in rats by the method described by Johnson (7). The individual bioassay data are shown in Table 1. Results were expressed in terms of DCA equivalents and converted to aldosterone by dividing by 36.5. Means were calculated for each group of animals and the significance of the differences between groups computed statistically, taking into account assay variability by analysis of variance.

RESULTS

The results of the sodium and potassium excretion studies in the two groups of animals with lesions are compared to the previously published results in similarly treated hypophysectomized and normal dogs in Tables 2 and 3. The values shown are expressed as dietary intake minus urinary excretion for each of these ions. The dogs with lesions in the median eminence excreted amounts of sodium and potassium which were not significantly different from the animals with lesions in other parts of the hypothalamus or from the normal dogs on the 40 mEq. diet. The two hypophysectomized dogs studied also showed normal values. Serum sodium and potassium values were similar in all groups. The decrease in urinary sodium excretion with salt restriction was normal in the animals with median eminence lesions, the animals with lesions elsewhere in the hypothalamus, and the hypophysectomized dogs. There was therefore no abnormality of sodium or potassium metabolism detected in the animals with lesions or the hypophysectomized dogs.

On the other hand, the aldosterone secretion values measured in the

TABLE 2. INTAKE MINUS URINARY OUTPUT AND SERUM LEVELS OF ELECTROLYTES IN DOGS
Diet: 40 mEq. sodium, 8 mEq. potassium

Preparation	Number of Animals	mEq./day Sodium		mEq./day Potassium		Serum Sodium	Serum Potassium
		Day 1	Day 2	Day 1	Day 2	mEq./l.	mEq./l.
Normal	15	+0.5±3.9*	−2.0±2.9	−8.8±1.2	−7.9±1.2	142	4.7
Median eminence lesions	6	+6.6±3.7	−2.8±9.7	−3.2±0.8	−6.2±2.0	146	5.1
Other hypothalamic lesions	6	+1.0±6.8	+2.1±2.1	−11.8±3.3	−8.6±2.7	142	4.8
Hypophysectomized	2	+1.2	+2.5	−12.9	−4.1	146	5.5

* Standard error of the mean.

TABLE 3. INTAKE MINUS URINARY OUTPUT AND SERUM LEVELS OF ELECTROLYTES IN DOGS (mEq./day ± standard error)

Diet: 0.5 mEq. sodium, 50 mEq. potassium

Preparation	Number of animals	mEq./day ± S.E. Sodium			mEq./day ± S.E. Potassium			Last day of diet	
		Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Serum Na mEq./l.	Serum K mEq./l.
Normal	15	−12.5±1.9	−5.6±0.7	−4.6±0.6	+2.7±2.2	+2.5±3.2	+1.5±3.6	145	5.0
Median eminence lesions	6	−15.2±4.8	−5.7±1.7	−3.2±0.6	+0.4±1.8	+2.0±2.1	+2.0±2.6	144	5.0
Other hypothalamic lesions	6	−11.7±2.8	−4.5±1.2	−2.2±0.4	+3.4±4.7	−5.1±5.0	−9.8±4.2	144	4.8
Hypophysectomized	2	−11.8	−5.8	−5.8	−2.6	−1.6	+2.0	147.5	5.2

adrenal venous blood show a slight but clear-cut difference between the dogs with lesions of the median eminence and the normal animals. The values obtained are plotted in Figure 1. The mean aldosterone secretion in the normal dogs of 3.5 ± 1.0[6] μg. per 2 hours compares with a value of only 1.5 ± 0.4 μg. in the dogs with lesions of the median eminence. The value in the dogs with other hypothalamic lesion was 3.7 ± 0.5 μg. per 2 hours. The difference between the dogs with median eminence lesions and the dogs

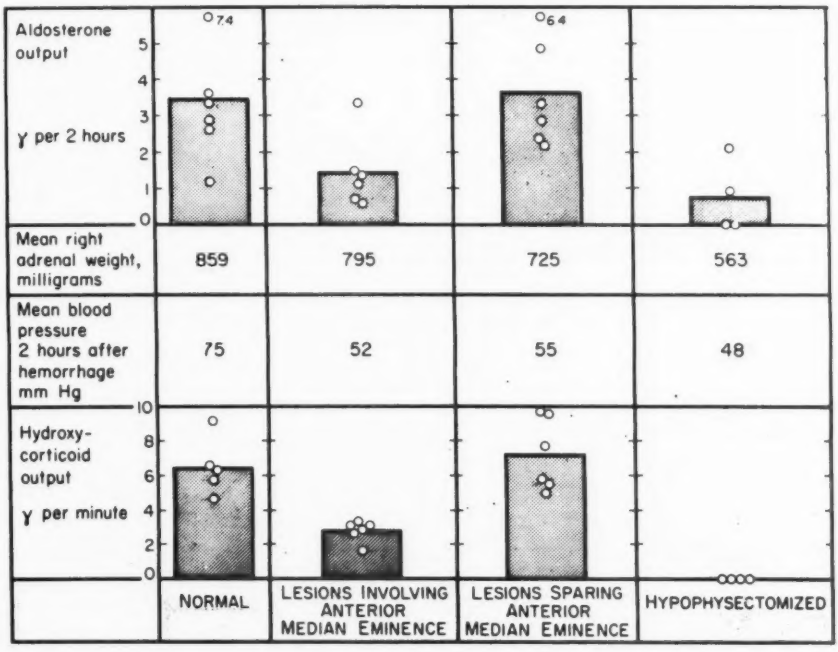

FIG. 1. Adrenocortical hormone output in dogs. The bars represent mean levels and the dots individual values.

[6] Standard error of the mean.

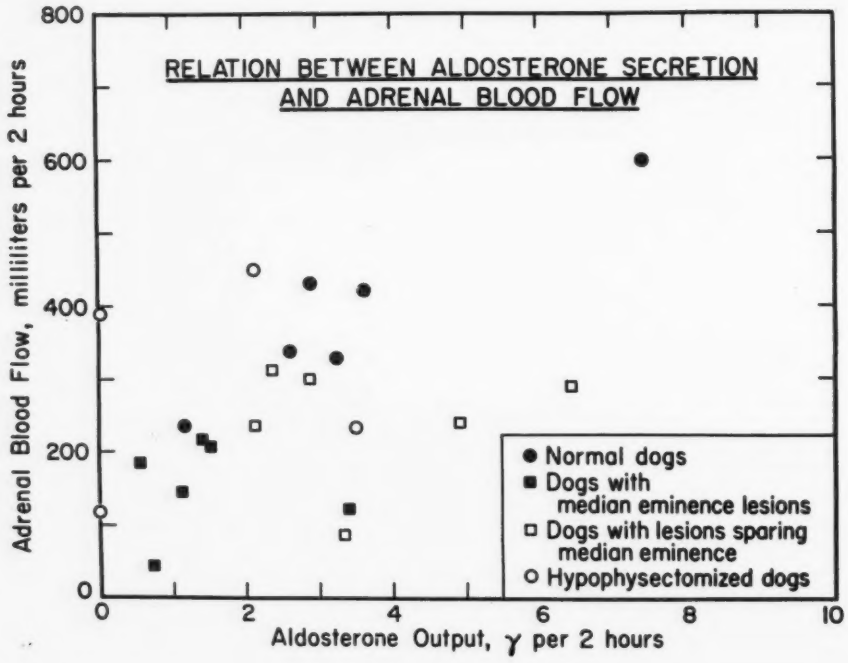

FIG. 2.

with lesions elsewhere in the hypothalamus is significant at the 5% level. The normal group includes one low value, but it may be stated with overall confidence of 90% that the mean response in group 2 is less than the average response of groups 1 and 3. The mean aldosterone output in the hypophysectomized dogs was 0.6 ± 0.5 μg. per 2 hours.

17-hydroxycorticoid secretion was also low in the animals with destruction of the median eminence, the mean value of 2.9 ± 0.3 μg./min. comparing to a value of 7.3 ± 0.9 μg./min. in the dogs with lesions in other parts of the hypothalamus and 6.5 ± 0.8 μg./min. in the normal controls. In the hypophysectomized dogs, the value was 0 in all cases.

The differences between the mean values for hormone secretion in all groups of animals were comparable to the above results when expressed in terms of micrograms per hundred kilograms of body weight per hour. The most uniform results were obtained when the output of aldosterone and 17-hydroxycorticoids were expressed in terms of micrograms per gram of adrenal per hour.

That the results obtained were not due to any differences in the blood pressure response to hemorrhage is shown in Figure 1. The mean blood pressure at the end of the two-hour bleeding period in the animals with lesions of the median eminence is considerably lower than that in the nor-

mal animals, but is not significantly different from that in animals with lesions in other parts of the hypothalamus, and the latter group of animals had normal values for aldosterone secretion. The diagram in Figure 2 shows that the results are not due solely to differences in the rate of adrenal blood flow. There is a rough correlation between the two-hour adrenal blood flow and aldosterone output, but there are exceptions, high aldosterone values being associated with low flow, and in the hypophysectomized dogs, low values with high flows. This rough correlation between flow and hormone output also holds true for hydroxycorticoid output in these experiments and in a variety of other experimental conditions (8).

The mean weight of the right adrenals in each group is shown in Figure 1. In terms of mg./kg. of body weight, the value in the dogs with lesions of

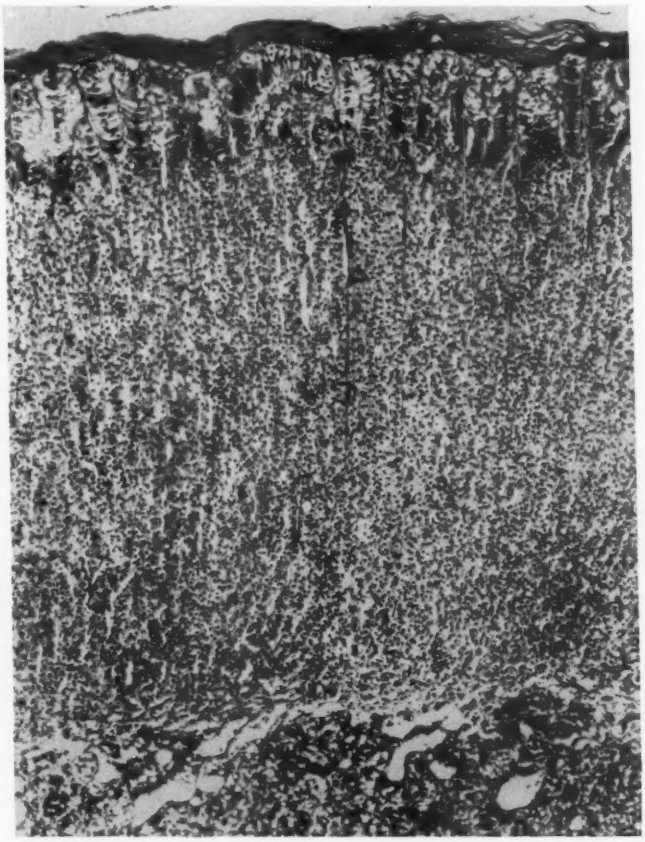

FIG. 3. Right adrenal, dog with hypothalamic lesion sparing anterior median eminence. Aldosterone secretion 3.3 μg./2 hrs. Hematoxylin and eosin. ×90.

the median eminence was 73.1 ± 11.1, and 54.3 ± 6.0 mg./kg. in dogs with lesions in other parts of the hypothalamus. This compares to a mean value of 58.5 ± 7.5 mg./kg. in the normal dogs with 40.3 ± 3.3 mg./kg. in the hypophysectomized dogs. The high value in the dogs with median eminence lesions is due to one very large value, and the mean is not significantly different from the normal statistically. Histologically, the zona glomerulosa of the adrenals of the dogs with lesions was indistinguishable from that of the normal dogs and there was no atrophy of the zona fasciculata (Figs. 3 and 4). In the hypophysectomized dogs, the zona fasciculata showed moderate to marked atrophy, but the zona glomerulosa was slightly more prominent than normal (Fig. 5). The kidneys were microscopically normal in all dogs.

Six of the dogs had diffuse gonadal atrophy on the basis of testicular

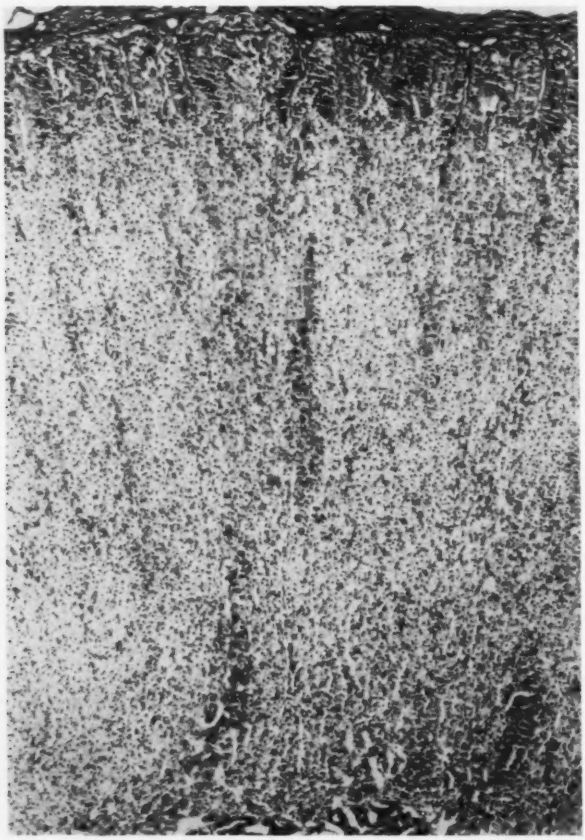

FIG. 4. Right adrenal, dog with destruction of anterior median eminence. Aldosterone secretion 1.4 μg./2 hrs. Hematoxylin and eosin. ×85.

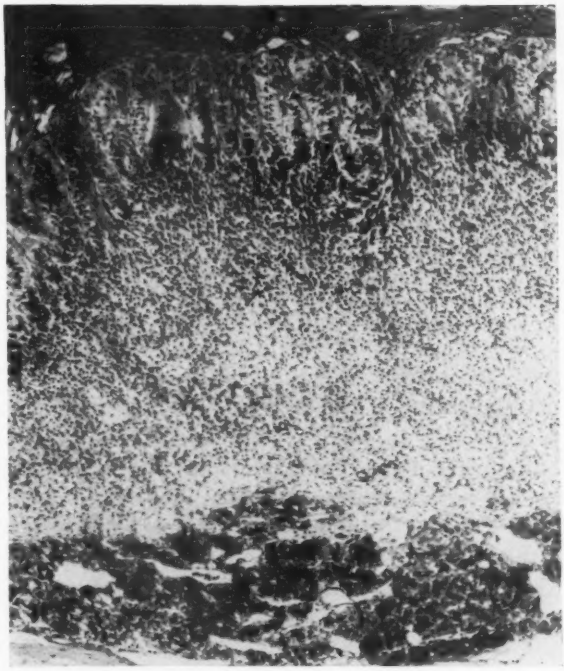

FIG. 5. Right adrenal, hypophysectomized dog. Aldosterone secretion 0 μg./2 hrs. Hematoxylin and eosin. ×80.

weight and histology. In three of these, aldosterone and 17-hydroxycorticoid secretion were low, and in the other three normal. Two of the dogs with lesions of the anterior median eminence had urine volumes in excess of 700 ml. per day on two consecutive days, but the remaining four dogs in this group did not have polyuria. The aldosterone secretion values there-

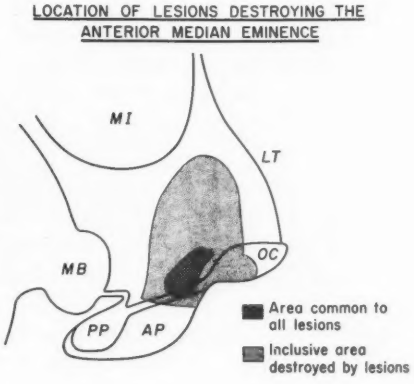

FIG. 6. Projection of hypothalamic lesions on mid-sagittal section of hypothalamus. MI, massa intermedia; MB, mammillary body; PP, posterior pituitary; AP, anterior pituitary; OC optic chiasm; LT, lamina terminalis.

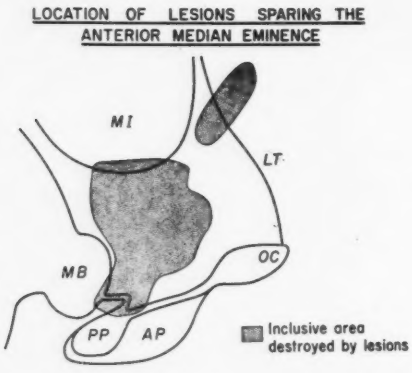

FIG. 7. Projection of hypothalamic lesions. See legend for Figure 6.

fore do not correlate with the presence or absence of diabetes insipidus or gonadal atrophy.

The sites of hypothalamic destruction in the dogs with lesions are shown schematically in Figures 6 and 7. The lesions involving the anterior median eminence included large portions of the anterior hypothalamus. The lesions sparing this area were in general more posterior, but none involved the posterior hypothalamus or the midbrain.

DISCUSSION

The normal values for serum and urinary sodium and potassium in dogs with hypothalamic lesions confirm the results of Daily and Ganong (3). As in that study, all animals with lesions withstood the low sodium, high potassium diet in a normal fashion. However, when their adrenal viens were cannulated and their aldosterone excretion measured directly, values in the dogs with median eminence lesions were lower than those in the normal dogs and in the dogs with lesions elsewhere.

One possible explanation of this result might be failure of the balance technique employed to reflect aldosterone deficiency. Daily and Ganong attempted to explore this possibility by subjecting adrenalectomized dogs on a 40 mEq. sodium diet to basal glucocorticoid maintenance therapy (5.0 mg. of hydrocortisone per day) without mineralocorticoid replacement. The series of three dogs previously published (3) has since been expanded to seven (8). In all instances, the dogs developed severe hyponatremia on a dietary intake of 40 mEq. of sodium per day. One animal died before being placed on the 0.5 mEq. sodium, 50 mEq. potassium diet and the remaining six animals died within 24 hours when subjected to this "metabolic stress." Accordingly, it seems reasonable to assume that if any appreciable aldosterone deficiency had been present in the dogs with lesions of the median eminence, these animals would have shown some hyponatremia and difficulty withstanding the low sodium, high potassium diet.

Another explanation is suggested by the correlation between low aldosterone secretion and low 17-hydroxycorticoid values. All animals in the

present series were subjected to the acute stress of adrenal vein cannulation prior to the measurement of aldosterone secretion. In the normal dogs, this causes a maximum secretion of 17-hydroxycorticoids (5, 9) and a rise in the blood ACTH (10). Is it possible, then, that the low aldosterone values in the animals with median eminence lesions can be explained on the basis of deficient endogenous ACTH secretion? If this hypothesis were true, one would expect hypophysectomy to lower the secretion of aldosterone by an amount roughly equal to the decrease seen in animals with lesions of the median eminence. Farrell, Rauschkolb and Royce (11) have previously reported that hypophysectomy in the dog leads acutely to a 30% decrease in aldosterone secretion, a decrease which persists for at least six days (12). Singer and Stack-Dunne (13), on the other hand, reported an 83% decrease in hypophysectomized rats 2 to 14 days after hypophysectomy. In the present experiments, the hypophysectomized dogs showed an average reduction in aldosterone secretion of 83% six weeks after hypophysectomy, while the percentage decline seen in animals with lesions of the median eminence was 56%. In the chronically hypophysectomized dogs, 17-hydroxycorticoid secretion was zero when measured by the method of Silber and Porter (6) on small amounts of adrenal venous plasma.

This hypothesis of an effect of endogenous ACTH on aldosterone secretion in the stressed dog finds support in the clinical literature. Llaurado (14) has shown that surgical stress in humans raises the level of aldosterone excreted in the urine. Hypophysectomy prevents this response (15), without abolishing the ability to change the level of secretion of aldosterone with changes in extracellular fluid volume (16, 17). There is no unanimity of opinion on the effect of injected ACTH on aldosterone secretion in man. However, most preparations of ACTH cause in normal humans a temporary rise in urinary aldosterone secretion, the value often falling back to the control level or lower after three to four days despite continued administration of corticotrophin (16, 17, 18). In hypophysectomized rats, Singer and Stack-Dunne (13) found that ACTH raises the depressed level of aldosterone secretion toward normal, and Farrell (19) has reported that one corticotrophin fraction is very active in causing aldosterone secretion in the decerebrate dog. Accordingly, it seems reasonable to conclude that aldosterone secretion is elevated in the acutely stressed animal, due at least in part to an action of the ACTH liberated by the stress. This effect is probably independent of the mechanism causing changes in aldosterone secretion with changes in dietary sodium and potassium. Further investigation of this concept would be of interest. Infusion of ACTH into the dogs with lesions of the median eminence would be of value, for instance, and another important experiment would be the measurement of aldosterone secretion in unstressed dogs. The latter experiment is technically difficult, but possible, using the technique of chronic adrenal vein cannulation as developed by Hume and Nelson (5).

In the meantime, it seems important to keep the present results in mind

in interpreting data on aldosterone secretion, as measured in adrenal venous blood. Most, if not all, of the published experiments have been performed on dogs subjected to acute adrenal venous cannulation. Since the cannulation procedure is a stress, the effect of increased ACTH secretion must be considered and results obtained in this fashion should not be generalized to conditions in the unstressed dog. The report by Newman, Yatsu, and Farrell (2) of decreased aldosterone secretion in the adrenal vein of cats after acute ventral hypothalamic lesions is probably explained on this basis. In other experiments, however, these investigators found a decrease in aldosterone secretion without changes in hydroxycorticoid secretion following acute destruction of portions of the mid-brain. Such differential secretion cannot be explained on the basis of ACTH secretion alone and further work on this portion of the brain is awaited with interest.

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STEROIDOGENIC PROPERTIES OF EXTRACTS OF BEEF DIENCEPHALON[1]

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ABSTRACT

Saline extracts of beef diencephalon were assayed in the decerebrate dog for steroidogenic activity. Acid saline extracts of whole diencephalon stimulated aldosterone and cortisol secretion. Neutral saline extracts prepared at room temperature were weakly active. However, neutral saline extracts of whole diencephalon prepared at 3° significantly stimulated aldosterone secretion without affecting the secretion of cortisol. Assay of extracts of parts of diencephalon demonstrated that the pineal complex contains the factor which stimulates the secretion of aldosterone.

THE physiological mechanism for the regulation of the adrenal secretion of electrolyte-active steroids has been the subject of interest for many years. Early evidence indicated that the output of electrolyte-active steroids is independent of that of the carbohydrate-active steroids (1, 2, 3). The discovery of aldosterone and the demonstration of its importance in electrolyte metabolism has stimulated reexamination of the problem. The concept of separate regulation has been strengthened by the finding that aldosterone secretion is well maintained following hypophysectomy in the dog (4) and the rat (5), and in patients with panhypopituitarism (6). Alterations in the rate of secretion of aldosterone occur in the absence of the pituitary (5). Further, the elaboration of aldosterone appears to be exempt from the homeostatic system for the control of cortisol secretion, since the injection of cortisone suppresses adrenal output of cortisol but not of aldosterone (7).

Final clarification of the physiological mechanism by which aldosterone secretion is regulated has thus far not been possible. However, evidence has been obtained that ablation of the diencephalon or lesions placed in the posterior diencephalon and anterior midbrain markedly reduce aldosterone secretion (8, 9, 10). It has been postulated that a humoral agent, for which the provisional name glomerulotropic hormone (G.T.H.) has been suggested, is released from the diencephalon to stimulate aldosterone secretion (11).

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[2] This work was done during the tenure of an Established Investigatorship of the American Heart Association.

A necessary step in the progression of the study is the assay of diencephalic tissue for the prsence of the postulated hormone. A preliminary report has been presented (12). In the present study saline extracts of beef diencephalon were assayed for steroidogenic properties. The results indicate that neutral saline extracts prepared in the cold stimulate aldosterone secretion but are free of ACTH-like activity. Extracts of the pineal complex appear to be particularly active in stimulating aldosterone secretion.

METHODS

Extracts of whole diencephalon. Beef brains were obtained fresh from the slaughterhouse[3] and kept in ice until use. A block of tissue corresponding to the diencephalon was removed as follows. An anterior cut was made on the frontal plane at the anterior border of the optic chiasm. A posterior cut was made on the frontal plane at the midcollicular level, sectioning the midbrain about 3 mm. caudal to the mammillary body. The pituitary was absent, having been removed at the slaughterhouse.

Three types of extracts were studied: 1) neutral saline, prepared at room temperature, 2) neutral saline, prepared in the cold room, and 3) acid saline, prepared at room temperature. In each instance the blocks of tissue, about fifty at a time, were homogenized for 30 seconds in a Waring blendor in 500 ml. of saline. The homogenate was diluted to 2 liters and centrifuged 15 minutes at 2000 r.p.m. The supernatant fluid (about 1600 cc.) was decanted and assayed for steroidogenic activity in the decerebrate dog.

In the preparation of extracts in the cold room, all materials were chilled to approximately 3°. The homogenate was frozen immediately after preparation, and subsequently thawed and centrifuged at 10° or less. The temperature of the final extracts varied between 4° and 8°. The solutions were warmed to room temperature just prior to injection. For preparation of acid saline extracts, the tissue was homogenized at room temperature in 0.9% NaCl made 0.1N with HCl, and 10N HCl was added dropwise to adjust to pH 2. The homogenate was centrifuged and the supernatant decanted for assay.

Extracts of parts of diencephalon. The block of brain tissue described above was further sectioned as follows. A cut was made on the frontal plane at the anterior border of the mammillary body, dividing the diencephalon into anterior and posterior portions. The pineal along with the attached anterior portion of the posterior commissure was removed from the dorsal portion. The three portions: anterior, posterior, and pineal region were homogenized and extracted as described above for the whole diencephalon. In some instances the pineal complex was ground in a mortar and pestle with sand, because of the small amount of tissue to be extracted. All extracts in this series of experiments were made in neutral saline in the cold.

The number of diencephalons or parts of diencephalon extracted and assayed in each assay dog varied somewhat from experiment to experiment. An effort was made to obtain fifty whole diencephalons, or parts equivalent to fifty, for each assay. In some instances the diencephalons had been cut in half during removal from the skull at the slaughterhouse, in which case the two halves were dissected into the indicated blocks of tissue and counted as one diencephalon. In the experiments in which the pineal complex was assayed separately it was not infrequently found that the pineal had been torn from the diencephalon in removal from the skull. For this reason additional pieces of pineal complex tissue were obtained from other diencephalons collected at the same

[3] We are grateful for the assistance of Mr. Gerald E. Kettering of Swift and Company, Cleveland, Ohio, in obtaining this material.

time, in order to have equal numbers of specimens. The wet weights of tissue extracted for assay in each experiment were as follows: fifty whole diencephalons, 700 to 900 gm.; anterior diencephalons and posterior diencephalons approximately equal weights, 350 to 450 gm.; pineal complex 3 to 5 gm.

Method of assay. In each experiment a large male dog was anesthetized with sodium pentobarbital. The carotid arteries were ligated. A flap of bone was raised on the dorsum of the skull, and the dura was resected to expose the occipital and parietal lobes of the cerebral cortex. The occipital lobes were removed with suction and the corpora quadrigemina were identified. The handle of a scalpel was passed through the brain stem at the midcollicular level. The brain substance rostral to the section and the pituitary gland were removed with suction. The internal carotid arteries and the posterior cerebral arteries were clamped inside the cranial cavity. Artificial respiration was routinely instituted by means of an alternating positive-pressure respirator. Periodic rectal temperature observations were made and heat lamps or ice packs applied as necessary to maintain body temperature within the range 36–40°. Arterial blood pressure was determined by means of a mercury manometer, and a solution of norepinephrine or epinephrine in 5% glucose was infused when necessary to maintain arterial blood pressure at or above 80 mm. Hg. Four hours after decerebration the left adrenal vein was cannulated for collection of adrenal venous blood. The solution to be assayed was infused by intravenous drip simultaneously with the adrenal venous blood collection, over a period of approximately 2 hours.

Cortisol and aldosterone diacetate were isolated by paper chromatographic methods described in detail in earlier reports (13). Quantitation was on the basis of absorption at 240 mμ. When the individual studies were completed, the samples of aldosterone diacetate obtained in the various experiments were pooled according to the type of extract studied, and rechromatographed to determine homogeneity. The pooled samples were then hydrolyzed and rechromatographed for the isolation of free aldosterone. The isolated steroid was assayed for electrolyte activity in adrenalectomized rats (14). Desoxycorticosterone acetate was used as the reference steroid. The potency of the various lots was found to be equal and consistent with that of aldosterone (approximately 60 times as potent as desoxycorticosterone acetate).

RESULTS AND DISCUSSION

The results of the experiments using whole diencephalons are summarized in Table 1. Acid saline extracts of diencephalon prepared at room temperature stimulated aldosterone secretion. However, cortisol secretion was also increased, indicating the presence of ACTH or ACTH-like material in such extracts. On the other hand, extracts made in neutral saline at room temperature (approximately 25°), while free of ACTH activity, were only weakly active in stimulating aldosterone secretion (Table 1). In an effort to avoid the presumed inactivation, neutral saline extracts were then prepared in the cold as described above (methods section). Highly significant stimulation of aldosterone secretion was obtained without any evidence of ACTH activity (Table 1).

This finding indicated that a substance which selectively stimulates aldosterone secretion is present in extracts of whole diencephalon. Assay of parts of diencephalic tissue was then carried out in an effort to determine in which part of the diencephalon the activity was present. In these experiments the extracts were prepared in neutral saline in the cold. The re-

TABLE 1. STEROIDOGENIC PROPERTIES OF SALINE EXTRACTS OF WHOLE BEEF DIENCEPHALON

Type of Preparation	No. of Experiments	Aldosterone Secretion μg./100 kg. body wt./hr.	Cortisol Secretion μg./kg. body wt./hr.
Controls*	6	8.5 ±0.95†	1.72±0.38
Acid saline extracts, 25°	4	37.3 ±7.4**	17.5 ±0.01**
Neutral saline extracts, 25°	4	14.1 ±2.2**	1.4 ±1.6 (N.S.)
Neutral saline extracts, 3°	7	24.21±3.2**	1.93±0.55 (N.S.)

* Saline infused.
† Mean and standard error.
** Statistically significant ($P < .01$) when compared with the corresponding controls. N.S. = not significantly different.

sults are presented in Table 2. As anticipated, no significant cortisol stimulating activity was found in extracts of any part of the diencephalon prepared in this way. On the other hand, a most interesting distribution of glomerulotropic activity was found. In comparing anterior diencephalon versus posterior diencephalon, it was evident that the posterior portion (with the pineal complex) contained the greater amount of activity (Table 2). When the pineal complex was removed and assayed in comparison with the remaining tissue from the posterior diencephalon (posterior diencephalon without pineal complex, Table 2), the greater amount of activity was found in extracts of the pineal complex. The difference in the biological activities of the extracts of the two types of tissue is highly significant ($P < .001$).

Although the present experiments were carried out on simple saline extracts, the results strongly support the concept that glomerulotropic hormone, a factor specific for the stimulation of aldosterone secretion, is present in the posterior diencephalon and may arise primarily from the pineal complex. Luetscher and his collaborators have evidence for a factor from human plasma and urine which selectively stimulates the synthesis of aldosterone by rat adrenal in vitro (15). It would be of considerable interest to determine if the substance studied by these workers is similar to that obtained from pineal extracts in the present study.

These observations add support to the concept that an area of the pos-

TABLE 2. STERIODOGENIC PROPERTIES OF NEUTRAL SALINE EXTRACTS OF REGIONS OF DIENCEPHALON*

Region extracted and assayed	No. of Experiments	Aldosterone Secretion μg./100 kg. body wt./hr.	Cortisol Secretion μg./kg. body wt./hr.
Whole diencephalon	7	24.2 ± 3.2	1.93±0.55
Anterior diencephalon	3	11.12± 3.0	1.06±0.19
Posterior diencephalon with pineal complex	2	25.2, 36.3	1.46, 1.20
Posterior diencephalon without pineal complex	6	12.5 ± 2.2**	2.15±0.95
Pineal complex	6	30.3 ± 2.9**	2.17±0.51

* Extracts were homogenized at 3° and the homogenates were frozen, later thawed, and centrifuged at 10°.
** P value <.001 in the comparison of these two groups.

terior diencephalon controls aldosterone secretion. It is of considerable interest that in recent experiments carried out in this laboratory, lesions in the central gray substance in the region of the posterior commissure have been found to significantly depress aldosterone secretion, again implicating the posterior diencephalon (and possibly the anterior midbrain) as important in aldosterone regulation (10).

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THE RESISTANCE OF THYROTROPHIC AND ADRENOCORTICOTROPHIC HORMONES TOWARDS PURIFIED THYROID AND ADRENAL PROTEINASES[1]

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ABSTRACT

The effect of incubation with purified proteinases from thyroid and adrenal tissue on the biological activity of TSH and ACTH has been investigated. It is shown that although TSH is inactivated by pepsin and by trypsin it is still functional after incubation, for a similar period, with either the thyroid or adrenal proteinase. ACTH was also resistant to the action of either enzyme.

These results are discussed with reference to the inactivation of TSH by thyroid tissue and ACTH by adrenal tissue and homogenates.

THYROTROPIC hormone (TSH) has been shown to be inactivated following incubation with pepsin or trypsin (1, 2). Adrenocorticotrophic hormone (ACTH) is also destroyed by trypsin (3, 4) as is the case with pepsin if the digestion is prolonged (5). According to Chow *et al.* (2) papain does not inactivate TSH, while Fels, Simpson, Sverdrup and Evans (6) found that activity was retained but that the hormone molecule was now able to pass through a dialysis membrane. ACTH, however, has been shown to be sensitive to this enzyme (7, 8) and also to pituitary proteinase (probably Proteinase II; (9, 10)).

Investigations in these laboratories have led to substantial purification of proteinases from the thyroid (11, 12) and adrenal (13) glands. The present studies have been concerned with the actions of these enzymes on preparations of TSH and ACTH in association with comparative experiments using pepsin and trypsin.

MATERIALS AND METHODS

The TSH used was a Parke Davis preparation from mixed hog and sheep pituitaries (batch No. 099802). It was found on assay by the acinar cell height method (chick) to contain 6–8 Junkmann-Schoeller (14) units/mg. The ACTH was a preparation[2] from pig pituitaries with a potency of 1 International Unit/mg.

The enzymes tested were crystalline pepsin and trypsin (Armour); thyroid proteinase with an activity of 429 units/mg.;[3] adrenal proteinase, purified by a procedure similar

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[2] Supplied through the courtesy of the Commonwealth Serum Laboratories, Melbourne.

[3] One unit is the amount of enzyme required to liberate 10^{-4} m.eq. tyrosine in 30 min. at 37° C. (haemoglobin substrate; pH 3.5); compare Anson (15).

to that used for thyroid proteinase (13), and with a potency of 305 units/mg.[3] At pH 3.5 the sample of pepsin was about four times as potent as the sample of thyroid proteinase against haemoglobin.

Generally, the hormones and enzymes were separately dissolved in the appropriate buffers and concentrations chosen so that equal volumes (1.0 ml.) were mixed prior to incubation. For the incubations with pepsin, thyroid and adrenal proteinases, the buffer solution was 0.02*M* NaAc (0.1*M* NaAc—Expt. 3 (Table 1) only) pH 3.5–3.6, and for trypsin, 0.025*M* sodium phosphate, pH 8.0, containing 0.01*M* $CaCl_2$. The latter enzyme was pre-incubated for 0.5 hour before the hormone solution was added. Control tubes contained (a) hormone alone and (b) buffer only. Incubations were carried out in tightly stoppered tubes for 16 hours at 37° C. (8 hours—Expt. 3 (Table 1) only), and the samples then stored in the frozen state prior to assay.

TSH activity was estimated by the increase in uptake of P^{32} by the thyroid glands of day-old cockerels (White Leghorn, Rhode Island Red crossbreed). The chicks were randomly allotted to groups—usually ten per group. One group acted as normal control and was injected with 0.25 ml. diluted buffer or isotonic saline. The other groups received 0.25 ml. of the diluted test or control solutions. In determining the dose of diluted solution, it was assumed that no change had occurred in the activity of the control (TSH alone) during incubation; the dose chosen was such that, in view of our experience with this assay, it would be expected to correspond to a point below the plateau of the dose-response curve. Any reduction in activity of test solutions would thus be correlated with the linear part of the curve. Two hours after the hormone injection 10 μc. P^{32} in water (0.25 ml.) was injected into all groups. All injections were intraperitoneal. The chicks were killed with ether 24 hours later and the thyroid glands dissected from one in each group in rotation. The glands, centrally placed on weighed planchettes, were allowed to dry overnight, weighed and counted with an EMH2 end window tube. The results are expressed as $10^{-5} \times$counts/min. multiplied by body weight (gm.) divided by thyroid weight (mg.). In the statistical treatment of the results the logarithms of the corrected counts were employed; thus any two means are statistically different if the difference between the logarithms of the values given is greater than $ts\sqrt{1/n_1+1/n_2}$, i.e. if their ratio is greater than antilog. $(ts\sqrt{1/n_1+1/n_2})$.

ACTH was assayed using intact mice according to a modification of a rat-assay method suggested by Professor E. B. Astwood. Adult male mice weighing between 20 and 25 gm. were pre-treated with 1 mg. of cortisone acetate suspended in 0.25 ml. saline injected subcutaneously 2 days and 1 day before use. The mice were then grouped randomly on a weight basis and the test and control solutions (0.25 ml. containing 16% gelatin) injected also subcutaneously. Four hours later the animals were killed with ether and each pair of adrenals dissected, weighed and ground with 4% trichloroacetic acid (10 ml.). Ascorbic acid determinations were carried out by the method of Roe and Keuther (16) and the results statistically analysed.

RESULTS

Effects on TSH

Pepsin. The test solution contained 1 mg. TSH (6–8 units) and 0.1 mg. pepsin each added in 1.0 ml. buffer (pH 3.5). Following incubation at 37° C. for 16 hours the test and control solutions were diluted to 10 ml. with distilled water. From the experimental and statistical results (Table 1, Expt. 1) it is clear that the hormone is inactivated by this enzyme ($P=0.01$; antilog.$(ts\sqrt{1/10+1/10})=1.373$).

Trypsin. In this experiment, activated trypsin (0.1 mg.) in the appropriate buffer (pH 8.0) replaced pepsin; otherwise the conditions were the same. The results are given in Table 1 (Expt. 2) and these show that trypsin also inactivates the hormone ($P = 0.01$; antilog.$(ts\sqrt{1/7+1/7}) = 1.910$).

Thyroid Proteinase. The results of two experiments are reported in which thyroid proteinase was tested against TSH. In the first experiment (Table 1, Expt. 3), TSH (1 mg.) in 0.1 ml. buffer (pH 3.5) was incubated for 8 hours at 37° C. with 0.1 mg. (Expt. 3a) and 0.5 mg. (Expt. 3b) thyroid proteinase, each amount being dissolved in 0.1 ml. buffer. Following in-

TABLE 1. ACTIVITY OF TSH FOLLOWING 16 HOUR INCUBATION AT 37° C. WITH PEPSIN, TRYPSIN, THYROID PROTEINASE AND ADRENAL PROTEINASE. P^{32} UPTAKE ASSAY ON CHICK THYROID

Expt. No.	Enzyme	Group 1 (0.25 ml. diluted buffer or saline)		Group 2 (TSH incubated control. 0.25 ml. diluted soln.)		Group 3 (TSH and enzyme. 0.25 ml. diluted soln.)		S.E.
		Counts*/min.	No. of chicks	Counts/min.	No. of chicks	Counts/min.	No. of chicks	
1	Pepsin 0.1 mg. enzyme to 1 mg. TSH	48.8	10	150.6	10	51.7	10	0.111
2	Trypsin 0.1 mg. enzyme to 1 mg. TSH	91.8	7	205.9	7	93.6	7	0.183
3**	Thyroid proteinase							
	(a) 0.1 mg. enzyme to 1 mg. TSH	45.8	10	100.0	8	97.6	8	0.184
	(b) 0.5 mg. enzyme to 1 mg. TSH	45.8	10	100.0	8	97.4	10	
4	Thyroid proteinase. Incubated solutions dialysed prior to assay.							
	(a) TSH solution (after dialysis)	48.8	10	150.6	10	125.1	10	0.110
	(b) dialysate	48.8	10	150.6	10	49.6	6	
5	Comparison of thyroid and adrenal proteinases							
	(a) thyroid 0.1 mg. enzyme to 1 mg. TSH	82.8	10	214.5	8	291.1	10	
	(b) adrenal 0.1 mg. enzyme to 1 mg. TSH	82.8	10	214.5	8	314.4	10	0.082
	(c) adrenal 1.0 mg. enzyme to 1 mg. TSH	82.8	10	214.5	8	223.0	10	

* Counts/min. are quoted as the geometric mean since logarithms of the counts were used in the statistical analysis. Counts can only be compared horizontally as the dose of P^{32} varied slightly; moreover the response of the chick's thyroid to a given dose of hormone shows a seasonal variation.
** 8 hour incubation only.

cubation the mixtures were diluted to 10 ml. with distilled water and assayed using 0.25 ml. per chick. TSH incubated alone served as a control. Examination of Table 1 (Expt. 3) leads to the conclusion that TSH activity is retained in the presence of thyroid proteinase, although the pH of the test was optimal for this enzyme (haemoglobin or serum albumin as substrate) ($P = 0.01$; antilog.$(ts\sqrt{1/10 + 1/8}) = 1.733$; antilog.$(ts\sqrt{1/8+1/8}) = 1.785$; antilog.$(ts\sqrt{1/10+1/10}) = 1.679$). In the second experiment (Table 1, Expt. 4), 10 mg. TSH in 10 ml. buffer (pH 3.5) was added to 1 mg. enzyme in 10 ml. buffer and the incubation period increased to 16 hours at 37° C. After incubation the mixture was dialysed, with stirring, against 250 ml. distilled water for 8 hours. The dialysate was then dried in the frozen state and dissolved in 3.0 ml. distilled water; the dialysed solution was diluted to 100 ml. with distilled water. Both solutions were then assayed at a dose of 0.25 ml. per chick. As can be seen from

Table 1 (Expt. 4a) the hormone has not been inactivated by the enzyme ($P=0.01$; antilog.$(ts\sqrt{1/10+1/10})=1.365$). Furthermore, in contrast to the reported action of papain (6), the hormone does not appear to have been degraded into active dialysable fragments (Expt. 4b; $P=0.01$; antilog.$(ts\sqrt{1/10+1/6})=1.433$); in fact, no loss in protein nitrogen could be detected after incubation and dialysis.

Adrenal Proteinase (including a comparison with thyroid proteinase). The adrenal enzyme was used at two levels (0.1 mg. and 1.0 mg. each in 1.0 ml. buffer (pH 3.5)). Thyroid proteinase (0.1 mg.) was dissolved in 1.0 ml. buffer while TSH (1.0 mg.) in 1.0 ml. buffer was added to the enzyme solutions and also acted as one of the controls. Incubation was for 16 hours at 37° C. The results are set out in Table 1 (Expt. 5) and indicate that TSH activity is retained after treatment with adrenal proteinase as with

TABLE 2. ACTIVITY OF ACTH FOLLOWING 16 HOUR INCUBATION WITH THYROID PROTEINASE OR WITH ADRENAL PROTEINASE AT pH 3.5 AND 37° C. ASCORBIC ACID DEPLETION ASSAY ON MICE ADRENALS

	Group 1 (16% gelatin only)	Group 2 (ACTH incubated control)		Group 3 (ACTH and thyroid proteinase)		Group 4 (ACTH and adrenal proteinase)	
		(a) 78 mU/ mouse	(b) 156 mU/ mouse	(a) 78 mU/ mouse	(b) 156 mU/ mouse	(a) 78 mU/ mouse	(b) 156 mU/ mouse
Number of mice	4	5	5	5	5	5	5
µg. ascorbic acid/10 mg. adrenal (mean)	31.8	22.4	10.2	16.6	9.8	21.4	10.0

The overall error mean square was calculated for all groups excluding Group 1, as this normal control group was only included in the experiment to provide an indication of response to the hormone. Overall S.E. = 3.99 with 24 d.f.

thyroid proteinase ($P=0.01$; antilog.$(ts\sqrt{1/10+1/10})=1.257$; antilog. $(ts\sqrt{1/10+1/8})=1.274$). In fact, there appears to be a highly significant increase in the assay values for Group 3 compared with Group 2 in the case of both enzymes. However, a definite conclusion on this latter point should be deferred pending further studies in which assays with more than single point comparisons are made.

Effects on ACTH

Table 2 shows the ascorbic acid content of the adrenals of cortisone-treated mice after injection of ACTH incubated alone or with thyroid proteinase or adrenal proteinase.

In this experiment, samples (5 mg.) of ACTH in 1 ml. buffer (pH 3.6) were incubated for 16 hours at 37° C. with 1 ml. buffer (Group 2), with 1 ml. thyroid proteinase (0.5 mg.; Group 3) or with 1 ml. adrenal proteinase (0.5 mg.; Group 4). The solutions were then diluted and assayed at two points for ACTH activity.

Analysis of variance showed that within the six groups treated with

hormone incubated alone or with either of the enzymes the response to the high dose was significantly different from the response to the low dose ($F = 48.48$ with 1 and 24 d.f.). No significant variation was found in the difference in response to the high and low dose in Groups 2, 3 and 4 ($F = 1.34$ with 2 and 24 d.f.), nor was there any significant difference in the level of the response to the high or to the low dose in any of these groups ($F = 1.70$ with 2 and 24 d.f.). This indicates that neither enzyme had affected the activity of the hormone.

The sample of ACTH used possessed melanocyte stimulating activity and preliminary studies of the effect of incubation with the two proteinases on the biological activity of this principle (technique of Landgrebe and Waring (17)) have indicated that there is no loss of activity and that there may be some increase. This effect is being further investigated.

DISCUSSION

Both TSH (18, 19) and ACTH (20) have been shown to be inactivated in the presence of tissue of the respective target gland. ACTH also loses activity when incubated with liver slices (21), liver and adrenal homogenates (21) and pituitary tissue once the cellular structure is broken (10). Our results, while confirming the previously observed sensitivity of TSH towards pepsin or trypsin (1, 2), also emphasize the surprising stability of both TSH and ACTH towards the proteinases, optimally active at acid pH, of either target gland. As regards TSH, this finding conforms to the conclusion of Rawson (19) that the inactivation of this hormone by thyroid tissue most likely involves an oxidative mechanism which is reversible. The nature of the process of inactivation of ACTH by adrenal tissue (20) or adrenal homogenates (21) has not been determined but, of the two pituitary proteinases, that optimally active at pH 8.3 is considered to be mainly responsible for the rapid loss of ACTH activity in aqueous extracts of fresh pituitary tissue (9, 10). It is of interest that ACTH is more sensitive to the action of trypsin than pepsin (5). It should also be noted in this connection that, although the purified sample of adrenal proteinase used in these investigations is inactive at alkaline pH, crude extracts of adrenal homogenates do exhibit moderate proteolytic activity at pH 8.0 (13). However, Li (21) observed that the supernatant fraction of adrenal homogenates, in contrast to the insoluble fraction, did not inactivate ACTH.

Hilliard and West (22) have described a pepsin inhibitor which is present in pituitary extracts and which is concentrated in some commercial TSH preparations. However, it is clear from Table 1 (Expts. 1 and 2) that both pepsin and trypsin destroyed the activity of the TSH preparation used in our experiments; moreover, it is of interest that both the thyroid and adrenal proteinases used, although ineffective as inactivators of TSH or ACTH, were shown to have retained full enzymic activity (at least 95%), with haemoglobin as substrate, after incubation with either hormone.

The indication of increased TSH activity following incubation with thyroid proteinase and with adrenal proteinase (Table 1, Expt. 5) deserves further investigation in view of the recent report by Dasgupta and Young (23) of the presence in pituitary extracts of inactive precursors of ACTH and prolactin. Similar precursors of TSH may also occur.

Acknowledgment

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A RADIOMIMETIC EFFECT OF TRIETHYLENEMELAMINE ON REPRODUCTION IN THE MALE RAT[1,2,3]

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ABSTRACT

The effect of triethylenemelamine on male rat fertility was investigated. Two dose levels were employed. A dose of 0.05 mg./kg. administered intraperitoneally five times each week for four weeks produced no histologically demonstrable effect on the germinal epithelium; however, it produced subfertility or infertility in the treated animals. The infertility was observed to be due to intrauterine death of fetuses in females inseminated by the treated males. There was also an indication that in some cases preimplantation death of the inseminated ova could have occurred. A dose of 0.2 mg./kg. daily for five days produced, in addition to the above-described effect on fertility, a destruction of spermatogonia followed by a maturation depletion of the germinal epithelium. The apparent similarity between the effects of triethylenemelamine and x-irradiation on the germinal cells is discussed.

DURING an investigation of the effect of various ethyleneimines on growth of neoplastic tissues, Hendry, *et al.* (1) observed that lethal doses of triethylenemelamine (TEM) produced severe, morphologically demonstrable damage to a number of tissues, including the germinal epithelium of the testes. Jackson and Bock (2) and Bock and Jackson (3) reported that TEM produced temporary sterility in male rats. The infertile animals, however, continued to produce morphologically normal-appearing, motile spermatozoa, and exhibited normal mating behavior.

The present paper deals with a segment of a study designed to elucidate the effect of TEM on the germinal epithelium of the male rat and the mechanism by which sterility is produced.

MATERIALS AND METHODS

Long-Evans rats raised at the State University of Iowa were employed for the preliminary experiments. All subsequent experiments were performed on a Long-Evans

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[3] Preliminary report of this work has been presented at the 1958 Annual Meeting of the Endocrine Society.

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strain of rats from a colony maintained at the Naval Medical Research Institute. The animals received "Rockland Rat Diet" and water *ad lib.* Male rats (250 ± 15 gm.) were divided into two groups. One group received, by the intraperitoneal route, 0.2 mg./kg. TEM[5] in 0.25 ml. normal saline solution daily for five or ten days (the high dose). The second group received 0.05 mg./kg. intraperitoneally five times each week for four weeks (the low dose). A fresh solution of TEM was prepared every 5 days and kept at 4° C. Control animals were injected with equivalent volumes of normal saline. The animals were sacrificed at various time intervals after termination of the treatment. The testes and sex accessories were weighed on a Roller-Smith torsion balance. The testes were fixed in Bouin's solution, embedded in paraffin, cut at 5 micra, and stained by the PAS-Weigert's hematoxylin method. For mating purposes, male rats were paired with normal females in individual cages. Each morning vaginal smears were examined microscopically for the presence of spermatozoa, Females with a positive sperm smear were considered to be inseminated; these were removed from the breeding cages and placed in individual maternity cages for further observation. The inseminated females were either sacrificed at various stages of gestation or permitted to complete pregnancy. In cases where young were delivered, they were nursed by the mother and observed until adulthood when they were mated and checked for ability to produce offspring. Autopsies were performed and reproductive organs inspected on all females sacrificed during the gestation period.

RESULTS

Preliminary experiments

Five male rats were treated with 0.05 mg./kg. TEM daily for 12 weeks beginning at age of 30 days. They were mated with normal females. Matings were observed on 24 occasions during this time and sperm plugs were found in 16 of these matings. However, no offspring was produced by the inseminated females.

Adult males were treated with 0.05 mg./kg. TEM for 30 days. Then they were allowed to recover for 7 days and were mated with normal adult females between the eighth and 16th day after termination of the treatment. Sperm plugs were found in 11 cases. Only one female delivered; the litter consisted of two normal young.

These experiments appeared to confirm the fact that TEM has a remarkable effect on germ cells. Subsequently the following studies were made.

Effect of TEM on organ weights and testicular morphology

a. Low dose series.

No effect could be detected on body, testicular and sex accessory weights (Table 1) following a four-week treatment with TEM as outlined above for the low dose series. Histological examination of testes from animals sacrificed from 5 to 75 days after termination of the treatment revealed normal spermatogenesis and normal appearing Ledyig cells.

[5] We wish to express our appreciation to Dr. J. M. Ruegsegger of the American Cyanamid Company for the generous supply of triethylenemelamine used in these investigations.

TABLE 1. EFFECT OF 0.05 MG./KG. TEM ON MALE RATS

Days after termination of treatment	No. of animals	Original body weight (gm.)	Final body weight (gm.)	Testes (mg.)	Seminal vesicles, full (mg.)	Seminal vesicles, empty (mg.)	Prostate (mg.)
5	3	239 ± 7.4*	240 ± 7.3	2662 ± 73.4	505 ± 37.5	263 ± 30.0	315 ± 19.5
Untreated controls	2	246 ± 5.5	248 ± 5.5	2681 ± 210.8	528 ± 61.0	258 ± 27.3	310 ± 22.1
26	8	242 ± 4.1	307 ± 8.2	2705 ± 63.2	617 ± 59.7	324 ± 38.1	393 ± 33.7
Untreated controls	6	250 ± 6.0	315 ± 10.1	2752 ± 89.2	600 ± 51.0	300 ± 23.7	410 ± 30.8
31	5	247 ± 4.5	327 ± 5.3	3050 ± 156.3	702 ± 73.6	287 ± 25.2	368 ± 30.8
Untreated controls	5	243 ± 3.1	318 ± 6.0	2920 ± 102.0	878 ± 58.1	393 ± 39.0	355 ± 20.0
75	19	246 ± 2.2	335 ± 8.1	3050 ± 59.0	689 ± 65.9	257 ± 22.7	377 ± 37.5
Untreated controls	20	251 ± 4.1	347 ± 5.0	3118 ± 115.1	701 ± 89.2	292 ± 29.0	363 ± 22.0

* Mean ± standard error.

b. High dose series (5 days' treatment).

Animals sacrificed 6 days after termination of treatment showed loss of body weight and decrease in testicular and accessory organ weights. They began to gain in body weight by the 17th day after termination of treatment and on the 30th day had caught up with and were gaining weight at the same rate as the control animals. The weights of sex accessory organs recovered fairly rapidly and were equal to those of control animals by the 17th day after termination of treatment. Testicular weights, on the other hand, continued to decrease progressively until the 36th day. By the 66th day post-treatment, they began to recover, and approached the normal weights 105 days after termination of treatment (Table 2). Histologic examination of the testes revealed a specific effect of treatment on the germinal epithelium. Seminiferous tubules of testes from animals sacrificed six days after termination of the treatment revealed either absence, or markedly decreased numbers, of spermatogonia. A definite decrease in numbers of the primary spermatocytes was evident in some tubules (Fig. 1). By the tenth day the majority of spermatocytes had disappeared, but spermato-

TABLE 2. EFFECT OF 0.2 MG./KG. TEM (FOR 5 DAYS) ON MALE RATS

Days after termination of treatment	No. of animals	Original body weight, gm.	Final body weight, gm.	Testes (mg.)	Seminal vesicles, mg. (full)	Seminal vesicles, mg. (empty)	Prostate (mg.)
6	3	257 ± 2.51*	240 ± 17.6	2372 ± 93.9	348.2 ± 66.6	191.0 ± 37.8	230.9 ± 34.8
Controls	2	242 ± 10.0	261 ± 14.9	2832 ± 65.1	525.0 ± 37.5	236.0 ± 37.9	389.6 ± 78.4
10	4	238 ± 3.3	221 ± 24.4	1982 ± 161.5	305.0 ± 122	153.3 ± 35.1	176.7 ± 34.9
Controls	3	243 ± 8.0	266 ± 6.2	2623 ± 102.0	511.3 ± 49.2	193.7 ± 20.4	224.0 ± 17.1
17	8	239 ± 2.1	259 ± 8.3	2262 ± 102.8	440 ± 43.5	214 ± 17.2	282 ± 18.9
Controls	6	251 ± 4.6	282 ± 9.1	3035 ± 157	446 ± 45.8	224 ± 19.1	269 ± 20.4
24	8	243 ± 3.6	256 ± 10.1	1655 ± 148.4	422 ± 43.2	214 ± 13.9	241 ± 22.7
Controls	4	245 ± 8.6	273 ± 17.8	2924 ± 120.9	520 ± 27.4	253 ± 7.9	297 ± 20.2
30	4	248 ± 1.2	298 ± 5.4	1757 ± 87.6	555 ± 170.1	257 ± 25.4	322 ± 36.9
Controls	2	245 ± 2.8	305 ± 6.9	2946 ± 207.9	509 ± 111.3	215 ± 9.0	305 ± 11.0
36	8	244 ± 3.9	293 ± 8.1	1470 ± 176.5	596 ± 48.8	248 ± 23.8	361 ± 13.1
Controls	2	251 ± 3.4	277 ± 11.2	2837 ± 110.0	448 ± 63.0	209 ± 15.2	332 ± 11.5
66	6	242 ± 3.7	291 ± 10.2	2343 ± 159.6	502 ± 74.0	249 ± 33.9	355 ± 40.9
Controls	2	245 ± 4.5	291 ± 6.9	2428 ± 163.9	534 ± 168.9	248 ± 22.6	369 ± 78.5
105	4	253 ± 4.7	361 ± 4.6	1690 ± 68.5	610 ± 71.5	281 ± 10.2	383 ± 11.3

* Mean ± standard error.

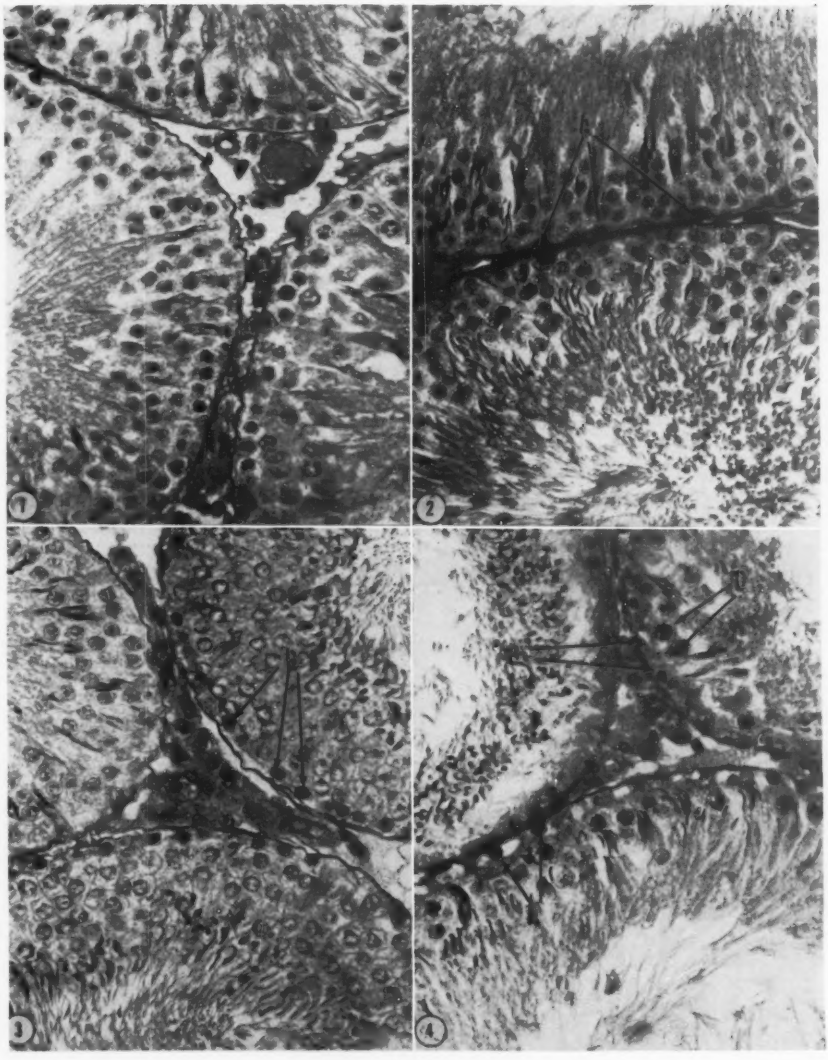

FIG. 1. Testes from a rat sacrificed 6 days after termination of treatment (0.2 mg./kg. daily for 5 days). Magnification ×300.

FIG. 2. Testes from a rat sacrificed 10 days after termination of treatment (0.2 mg./kg. daily for 5 days). Magnification ×300.

FIG. 3. Testes from a rat sacrificed 17 days after termination of treatment (0.2 mg./kg. daily for 5 days). Magnification ×300.

FIG. 4. Testes from a rat sacrificed 24 days after termination of treatment (0.2 mg./kg. daily for 5 days). Magnification ×300.

A—Spermatogonia
B—Resting spermatocytes
C—Pachytene spermatocytes

gonia began to increase in numbers (Fig. 2). Further increase in the number of spermatogonia and reappearance of resting spermatocytes was observed 17 days after termination of treatment (Fig. 3). After the 24th day pachytene spermatocytes reappeared and resting spermatocytes increased in numbers (Fig. 4). Further recovery proceeded with appearance of spermatids in many tubules of the testes from rats sacrificed on the 36th day (Fig. 5). Complete recovery of spermatogenesis was evident in animals sacrificed 67 days after termination of treatment (Fig. 6).

Effect of TEM on fertility

a. Low dose series.

The data of this experiment, summarized in Table 3, reveal that in pregnancies resulting from mating in the period between the 5th and 18th days after termination of treatment, fetuses were found infrequently and in small numbers of the uteri of pregnant females. Between the 18th and 39th days, the number of fetuses per mother increased gradually, but remained below the numbers observed in the females mated with control

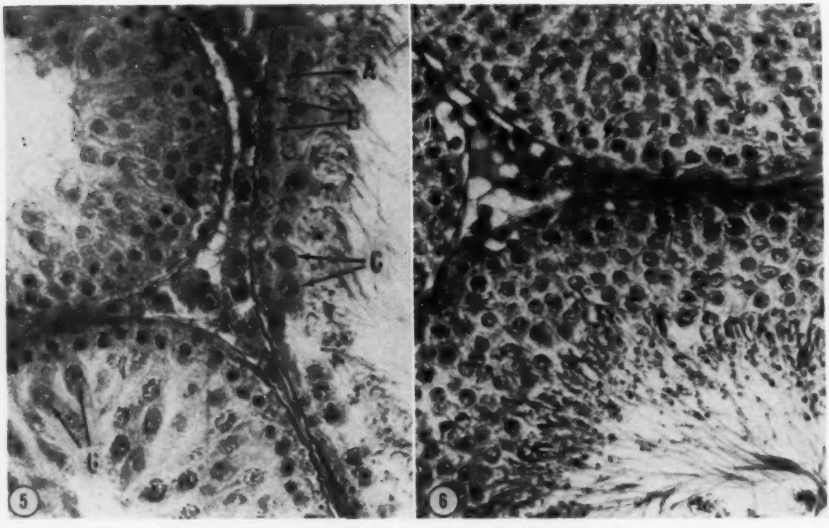

FIG. 5. Testes from a rat sacrificed 36 days after termination of treatment (0.2 mg./kg. daily for 5 days). Magnification ×280.

A—Spermatogonia
B—Resting spermatocytes
C—Pachytene spermatocytes

FIG. 6. Testes from a rat sacrificed 67 days after termination of treatment (0.2 mg./kg. daily for 5 days). Magnification ×280.

A—Spermatogonia
B—Resting spermatocytes
C—Pachytene spermatocytes

TABLE 3. EFFECT OF 0.05 MG./KG. TEM ON FERTILITY OF MALE RATS

Number of Impregnated Females and their Respective Litter* Sizes at Various Periods after Termination of Treatment

Days after termination of treatment	Litter size														Total No. of females	Mean litter size
	0	1	2	3	4	5	6	7	8	9	10	11	12	13		
6– 8	2	3		1											6	1.0
9–11	3	1	1												5	0.6
12–14	5	2	2	2		2	1								14	2.0
15–17	1			1	1			1							4	3.2
18–20	1				1	3	1			1					7	4.8
21–23	1			1					1	1	1				5	6.0
24–26		1	1			1	2	1	1	3					10	6.2
27–29			1		3	1	1		1						7	4.8
30–32			2		1	1				1					5	4.4
33–38				1	1					2					4	6.2
39–44							2		4	4	3	1	1	1	16	9.1
45–55							1	6	3	4	3	1			18	8.3
56–70					1	2	1	1	3	5	3		3		19	8.6
Untreated Males						1	1	3	6	2	1	3	1		18	8.5

* The expression "litter" is used in this table to denote fetuses found upon autopsy as well as normally delivered young.

males. After the 40th day, the number of fetuses per mother was comparable to that of the control group. Autopsy of females during the gestation period showed that in pregnancies resulting from mating with males up to the 24th day after termination of TEM treatment, all fetuses exhibited grossly demonstrable evidence of resorption; in pregancies resulting from mating with males between the 24th and 40th days after termination of treatment, some fetuses were undergoing resorption while others appeared to be developing in a normal manner. In pregnancies resulting from mating with males 40 days post-treatment all or most of the fetuses appeared normal. Offspring of mothers who were permitted to deliver reached adulthood and appeared normal.

b. High dose series.

Five days' treatment

Data summarized in Table 4 reveal a pattern of temporary subfertility

TABLE 4. EFFECT OF FIVE DAILY DOSES OF 0.2 MG./KG. TEM ON FERTILITY OF MALE RATS

Number of Impregnated Females and Their Respective Litter* Sizes at Various Periods after Termination of Treatment

Days after termination of treatment	Litter size														Total No. of females	Mean litter size
	0	1	2	3	4	5	6	7	8	9	10	11	12	13		
10–12	3		1												4	0.5
13–15	4	1	1												6	0.5
16–18	7	3	1	2											13	0.8
19–21	6		1												7	0.3
22–24		2					1								11	0.7
25–27	2	4	2	2		1		1							13	2.5
28–30	1	1		1			1	1							6	4.5
31–33	1	2	1					2		1			1		6	6.0
34–36	1					1							1		3	5.6
37–39					1				1						2	6.0
40–42	1								1				1		3	6.6
43–45					1						1				2	7.0
46–48							1					2	1		4	10.0
Untreated males							2	1	1	5	1	1	1	2	14	9.9

* The expression "litter" is used in this table to denote fetuses found upon autopsy as well as normally delivered young.

similar to that described for the low dose series. None, or very few fetuses were found in the uteri of females fertilized by males up to the 24th day after termination of treatment, a marked increase in numbers of fetuses was found in pregnancies resulting from mating between the 24th and 40th day after treatment, and after the 40th day the number of fetuses approached the control values. The resorption pattern was also similar to that observed in the low dose series. Up to the 24th day, all fetuses were resorbing; between the 24th and 40th days after treatment, evidence of resorption was observed less frequently; and after the 45th day, all fetuses appeared normal and animals permitted to go through the gestation period delivered normal-appearing litters. These offspring were observed through adulthood and mated; animals of the second generation were found to be apparently normal. The second generation animals were also mated and their offspring were found to appear normal.

TABLE 5. EFFECT OF TEN DAILY DOSES OF 0.2 MG/KG. TEM ON FERTILITY OF MALE RATS

Number of impregnated females and their respective litter* sizes at various periods after termination of treatment

Days after termination of treatment	Litter Size												Total No. of Females	Mean litter size
	0	1	2	3	4	5	6	7	8	9	10	11		
6–14	3	3											6	0.5
15–60	9	3											12	0.2
61–73				1	1								2	3.5
90–95								1	2	1		3	7	9.3

* The expression "litter" is used in this table to denote fetuses found upon autopsy as well as normally delivered young.

Ten days' treatment

A group of male rats treated with the high dose of TEM for ten days showed an appreciably longer period of subfertility than was seen in animals treated for five days (Table 5). Up to the 60th day after treatment, all matings resulted in pregnancies with no or at most very few resorbing fetuses. Mating between the 61st and 73rd days post-treatment resulted in a small number of resorbing fetuses. Between the 73rd and 90th days no inseminations were observed although the males were mated in the same way as throughout the entire experiment. After 90 days there was a sudden return of completely fertile matings and litters of normal size were produced with no evidence of resorption. Such females as were permitted to deliver had normal-sized litters and these offspring were shown to be fertile on subsequent matings.

DISCUSSION

The data presented in this paper indicate that TEM, depending on the dose employed, has a dual effect on the germinal epithelium of the male rat. The low dose, 0.05 mg./kg., does not interfere with spermatogenesis as

far as the production of spermatozoa and the morphologic integrity of the seminiferous epithelium is concerned. However, it produces a subtle effect on the germinal cells, including the mature spermatozoa. Morphologically normal appearing spermatozoa with fertilizing power and ability to initiate the development of the ovum are produced, but the development of ova fertilized by such sperm does not proceed in a normal manner, death and resorption of the embryos occurring early in gestation.

The high dose, 0.2 mg./kg. for five days, produces similar subtle effects on the germinal cells, including probably the spermatozoa since only a few days after termination of treatment the males are unable to produce normal pregnancies. Our experiments provide no data to explain the mechanism of this subtle effect on the germinal epithelium and spermatozoa. TEM is one of the alkylating agents. Recent *in vitro* studies on the mechanism of action of alkylating agents on biological material suggest that the effect is probably produced by a chemical reaction involving esterification of the phosphate groups of the DNA molecules, crosslinking and formation of a gel (4 and 5). Whether this or similar biochemical processes is the basis for the effect of TEM on the germinal epithelium can be only conjectured.

The high dose of TEM has also a specific, morphologically demonstrable effect on the germinal epithelium. Histologic studies of the testes showed that at this dose level TEM has a definite effect on the spermatogonia. Following the disappearance of spermatogonia maturation depletion of the germinal epithelium takes place. The weight of the sex accessories of the experimental animals suggests that the effect of TEM on the germinal epithelium probably is not mediated via an endocrine path, at least the changes apparently are not due to a lack of androgenic hormone.

Spitz (6) reported testicular degeneration in men treated with nitrogen mustard. Landing, *et al.* (7) and Kindred (8) demonstrated that alkylating agents of the nitrogen mustard class produce testicular damage in experimental animals. Kindred observed severe damage to the germinal epithelium of rat testes within 5 days after a single injection of nitrogen mustard. The damage was not localized to any particular type of cells. Spermatozoa were "coagulated," spermatids showed pyknosis, karyolysis and formation of giant cells; this picture was associated with changes in the spermatocytes and spermatogonia. Most severely affected testes revealed tubules containing only Sertoli cells and spermatogonia. Our studies with TEM reveal that this compound can produce a morphologic effect on the spermatogonia followed by a rather orderly maturation depletion of the remaining seminiferous epithelium. There is no reason to believe that other alkylating agents like nitrogen mustard would not produce a picture similar to that following TEM treatment if they were administered at appropriate dose levels.

It has been generally accepted that ionizing radiation is capable of pro-

ducing the picture of spermatogonial damage followed by maturation depletion of the germinal epithelium. There is, however, a difference between the effect of TEM on spermatogenesis and that of X-irradiation. Following a moderate dose of X-irradiation (500 r total body irradiation or 600 r local dose) a similar type of depopulation of the tubules occurs which results in almost complete denudation of the tubules in about 4 weeks after exposure (9 and 10). Repopulation of the tubules does not begin until 4 to 5 weeks after the exposure and according to Shaver 4 to 5 months are required for complete recovery. Following a 5-day treatment with 0.2 mg./kg. TEM recovery is already evident ten days after termination of treatment and in 36 days many of the tubules contain pachytene primary spermatocytes. Data available at this time do not provide enough information to explain this difference in the rate of recovery. The most likely hypothesis would be one taking into account the possibility of a dose difference. This is made more plausible by the experiment showing that a 10-day treatment with 0.2 mg./kg. TEM produces an effect on fertility similar in the time relationships to that described for 500 r total body irradiation, a period of subfertility followed by sterility and lasting for about 3 months after termination of treatment.

Using one parameter, namely, the number of young delivered by normal female rats mated with TEM-treated males, Bock and Jackson (3) concluded that TEM has a selective effect on the germinal epithelium, affecting the spermatogonia. This conclusion was based mainly on data showing that following a single injection of 0.2 mg./kg. TEM the males became subfertile for three weeks, sterile during the fourth week and fertile again 30 to 39 days after the injection. On the basis of Shaver's (9) work showing that about 27 days are required for formation of mature spermatozoa from spermatogonia they concluded that the time necessary for production of sterility after a single injection of TEM corresponds to the time that would have been necessary for a spermatogonium affected by TEM to become a mature sperm. In a later paper (11) an opinion is expressed that this conclusion was incorrect because the duration of the epididymal phase (12–16 days, according to MacMillan and Harrison (12)) was not taken into consideration. In the same paper they express the opinion that "the infertility in the fourth week after giving TEM appears to be the result of interference with a later stage of spermatogenesis." They do not attempt to identify this stage.

The data presented in our paper demonstrate that after 5 days of treatment with 0.2 mg./kg. TEM there is a definite morphologic effect on the spermatogonia. Quantitative investigation of the germinal epithelium of rats given a single dose of 0.2 mg./kg. TEM, presently in progress, also reveals definite morphologic damage to the spermatogonia. However, the sterility following a single dose of TEM is more likely to be a result of a

subtle effect of TEM on all or the more mature germinal cells rather than an effect on spermatogonia specifically. This would appear to be true since following a single injection of TEM the number of spermatozoa which would have originated from the affected spermatogonia would be a small percentage of the total number at a given time, being diluted by presumably normal spermatozoa already present in the epididymis as well as by new spermatozoa arising from the next generation of spermatogonia.

In our experience, doses of 0.2 mg. TEM per kg. for 5 days or 0.05 mg per kg. for 30 days failed to produce a condition in which sperm were completely incapable of fertilizing ova. This was demonstrated by the finding that many females sacrificed during gestation showed one or more conceptuses. In the group of females fertilized by males treated with the low dose of TEM, all pregnancies conceived up to 24 days after termination of treatment revealed either absence or resorption of fetuses. This corresponds to the 30-day period of sterility reported by Bock and Jackson (3) after low dose TEM treatment. Between the 30th and 40th day after termination of treatment, Bock and Jackson observed subfertility expressed by a small size of litters. This corresponds to the period of time where we observed pregnancies in which some of the fetuses were resorbing while others were developing normally. Pregnancies conceived after the 40th day reached term and resulted in delivery of normal litters in our experiments as well as in those of Bock and Jackson. A similar pattern was observed in experiments in which the males were treated with a high dose of TEM for 5 days.

Histologic study of the resorbing fetuses, presently in progress, may shed some light on the mechanism responsible for fetal death. It is of interest to note that shortly after exposure to X-irradiation, in the period of time preceding sterility, male rats become subfertile. Autopsies of pregnant females fertilized by these males also reveal fetal resorption (13).

Following treatment with 0.2 mg./kg. for 10 days, the period of subfertility is markedly prolonged and is succeeded by a period of sterility associated, apparently, with azoospermia. The period of sterility is terminated by an abrupt return to an essentially normal degree of fertility. This pattern could be explained by assuming that a complete maturation depletion, similar to that following an exposure to a "moderate" dose of X-irradiation, takes place, and that the sperm which effect fertilization are those formed during recovery, the older TEM-affected sperm having disappeared or being no longer able to effect fertilization.

Spermatozoa of TEM-treated males are capable of reaching and penetrating the ovum (3). Our data reveal that some fertilized ova apparently do implant, but are unable to proceed with a normal development, and resorption of the conceptus occurs. Indeed it is possible that in some instances sperm penetration takes place, but syngamy fails to occur. Such ova may

undergo an abortive form of cleavage, as has been observed in studies of sea urchin eggs fertilized with X-irradiated sperm. A careful investigation of fertilized tubal ova may provide morphological evidence for preimplantational degeneration.

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ACTION OF ESTROGEN AND PROGESTERONE ON MAMMARY NUCLEIC ACIDS AND ENZYMES IN RATS[1]

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ABSTRACT

Immature ovariectomized rats were injected daily for ten days with estradiol benzoate, progesterone, or vehicle, alone or in combination. Mammary glands were then removed for histological study and analyzed for per cent dry weight (DW), defatted dry weight (DDW), nitrogen (TN), nucleic acids (DNA and RNA), and enzymatic activity. Succinoxidase and cytochrome oxidase were measured manometrically, and arginase was assayed by colorimetric estimation of the urea formed.

Statistical evaluation revealed that neither estrogen nor progesterone given singly led to significant elevation in any of these mammary constituents compared to those of untreated control animals. Increments, however, were brought about by combined treatment, arginase showing the most striking rise. This increase was greater than the elevation in DNA concentration, indicating increased activity per cell. Augmentation of arginase activity, as well as of other constituents, was correlated with lobulo-alveolar development.

It is proposed on the basis of these data and those in the literature that in animals suitably prepared with estrogen and progesterone, elevation in succinoxidase-cytochrome oxidase and in arginase might serve as endpoints for lactogenic and lactopoetic assay, respectively, of hormone preparations.

SEVERAL studies have shown that progesterone synergizes with estrogen in producing lobulo-alveolar development in the mammary glands of rats (1, 2, 3), rabbits (4, 5, 6), mice (7) and guinea pigs (8, 9). Synergism has been indicated by histological evidence of enhanced lobulation (1, 3, 4, 5, 7, 9) and, more recently, by increased nucleic acid content (2, 6, 9, 10). No experiments have been reported, however, in which weight, chemical composition, and metabolic activity, as well as histological changes have been compared in a single study in rats. A similar study, however, has been reported in guinea pigs (9). Since chemical or metabolic data may more readily be expressed quantitatively than histological appearance of lobulo-alveolar development, these criteria might therefore serve as a basis for assay of mammary growth-inducing activity of various hormones.

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Mammary glands of immature ovariectomized rats were considered suitable for this purpose since their response to a range of estrogen and progestrone doses, as measured by morphological criteria, has previously been described (3). In that study, it was concluded that a ratio for maximal lobulation might be approached by the administration of 1 μg. estradiol and 3 to 5 mg. progesterone daily.

MATERIALS AND METHODS

Immature rats of the Harvard colony were ovariectomized. After a few days for recovery, they were injected daily for 10 days with either 1 μg. estradiol benzoate or 5 mg. progesterone, or a combination of the two hormones, each in 0.1 ml. sesame oil.[3] Treatment groups were caged separately. The day after the last injection the animals were stunned, decapitated and the abdominal-inguinal glands quickly excised and removed to ice-cold beakers and weighed. Samples for histological study were fixed in either neutral buffered formalin or Zenker-formol. The remaining tissue was weighed and homogenized with isotonic KCl in a conical ground-glass tube and assayed immediately for succinoxidase and cytochrome oxidase by the method of Schneider and Potter (11) and for arginase by the procedure of Folley and Greenbaum (12). Nitrogen was analyzed by Nesslerization of an aliquot of digested homogenate. Nucleic acids were determined on another aliquot by trichloracetic acid fractionation (13), with colorimetric estimation of the deoxyribose (14) and ribose (15) in the extract. Dry weight was obtained by heating a measured volume of homogenate overnight at 110° C and defatted dry weight then determined on the same samples after triple extraction with acetone at room temperature and redrying in the oven for an hour or more.

Differences between the various treatments have been evaluated statistically with "Student's" t test. Probability values less than 5% have been considered significant; those less than 1% highly significant; and those less than 10% merely suggestive. Concentrations of certain mammary constituents have also been compared with those of pregnant and lactating rats reported in a previously published study (16).

RESULTS

Neither estrogen nor progesterone, given singly, brought about a change in the per cent dry weight (DW), defatted dry weight (DDW), nucleic acid (DNA and RNA), or nitrogen (TN) content of the mammary gland, when compared to glands from rats given vehicle alone (Table 1). A combination of estrogen and progesterone, however, led to an enhanced concentration of all these constituents. Statistically, the combination was more effective than progesterone alone in increasing per cent dry weight and ribonucleic acid (RNA) concentration, and more effective than estrogen alone in increasing per cent defatted dry weight, nucleic acids, and nitrogen. There were no significant differences between estrogen and progesterone treatments. When the concentration of RNA was expressed relative to that of DNA, a rather consistent trend was revealed among the various treatments (Table 2). The combination treatment was more effective than either estrogen or progesterone alone in increasing the ratio, RNA/DNA, but no such trend could be observed in the ratio TN/DNA. Enhancement of

[3] The estradiol benzoate was generously supplied by Ciba Pharmaceutical Products, and the progesterone by Schering Corporation.

TABLE 1. COMPOSITION OF RAT MAMMARY GLANDS AFTER VARIOUS TREATMENTS

Treatment	Number of groups	Per cent fresh weight				
		DW	DDW	DNA	RNA	TN
I. Vehicle	5	29.4±4.6	11.1±1.1	0.47±0.11	0.06±0.02	0.88±0.20
II. Progesterone	6	27.5±1.5	12.7±1.3	0.59±0.12	0.10±0.02	0.96±0.18
Probability II→IV		<0.05	N.S.	N.S.	<0.02	N.S.
III. Estrogen	10	32.3±2.6	11.8±1.1	0.47±0.08	0.09±0.01	0.88±0.13
Probability III→IV		N.S.	<0.05	<0.05	<0.02	<0.05
IV. Estrogen and progesterone	8	37.2±1.6	16.2±1.6	0.86±0.14	0.20±0.03	1.38±0.18

Values are means ±standard errors

$$\text{S. E.} = \sqrt{\frac{\text{Variance}}{n}}$$

$$t = \frac{\text{Difference between means}}{\text{Standard error of difference}}$$

Probability values from Fisher and Yates t table
N.S. = not significant

Disposition of rats was as follows:
Group I, 1–3 (average 2) per experimental group
Group II, 1–3 (average 2) per experimental group
Group III, 1–3 (average 2) per experimental group
Group IV, 1–2 (average 1.5) per experimental group

nucleic acids, as well as of the other mammary constituents by the combination of estrogen and progesterone was observed consistently throughout the series of experiments.

Mammary glands of rats receiving progesterone or estrogen showed no marked differences in either succinoxidase or cytochrome oxidase from glands of control rats (Table 3). Animals receiving both hormones together showed a marked increase in succinoxidase per milligram fresh or defatted dry weight and in cytochrome oxidase per milligram fresh weight. Activity per milligram deoxyribonucleic acid was unchanged by the combination treatment, suggesting that enzyme levels keep pace with cellular proliferation. Under the same conditions, content of cytochrome oxidase lagged behind that of ribonucleic acid, so that activity decreased on this basis.

The elevation in arginase activity in glands from rats treated with the combination of estrogen and progesterone was the most striking change seen in these experiments (Table 4). This was apparent on all bases, although only three have been used for illustration: fresh weight, total nitro-

TABLE 2. RATIO OF MAMMARY CONSTITUENTS

Treatment	RNA/DNA	TN/DNA
Vehicle	0.15±0.11	1.92±0.18
Progesterone	0.19±0.03	1.65±0.06
Estrogen	0.22±0.03	1.90±0.08
Estrogen and Progesterone	0.26±0.03	1.57±0.10

TABLE 3. MAMMARY SUCCINOXIDASE AND CYTOCHROME OXIDASE OF EXPERIMENTALLY-DEVELOPED GLANDS

Treatment	Number of Groups	Succinoxidase (μlO_2/hr./mg.)				Cytochrome Oxidase (μlO_2/hr./mg.)			
		FW	DDW	DNA	RNA	FW	DDW	DNA	RNA
I. Vehicle	4	0.3±0.07	2.7±0.3	64±6	399±24	3.9±0.7	33.3±2.4	835±108	5121±367
II. Progesterone	3	0.3±0.07	2.3±0.6	52±18	303±93	4.2±0.4	37.8±10.5	893±411	4800±1800
Prob. of diff. II→IV		<0.01	<0.02	N.S.	N.S.	<0.01	N.S.	N.S.	N.S.
III. Estrogen	10	0.3±0.04	2.7±0.2	76±11	355±35	3.8±1.0	33.5±2.9	934±56	4171±263
Prob. of diff. III→IV		<0.001	<0.01	N.S.	N.S.	<0.01	N.S.	N.S.	<0.02
IV. Estrogen and Progesterone	8	0.8±0.11	4.6±0.5	95±17	395±45	6.1±0.6	37.8±0.5	799±34	3353±39

gen, and deoxyribonucleic acid. Either hormone alone failed to effect any change in activity.

DISCUSSION

Histological study of mammary glands used in the present experiments confirmed observations made on whole mounts in a preceding study (3) that estrogen alone elicited primarily ductal growth, progesterone alone had only slight effect, while the combination of the two hormones brought about marked ductal and lobulo-alveolar development. It is, therefore, pertinent to inquire whether such glands, morphologically similar to those of primiparous rats, compare with them biochemically.

Per cent DNA, nitrogen, or defatted dry weight of glands developed by either estrogen or progesterone resemble those of middle or late pregnancy, while those developed by the combined treatment of estrogen and progesterone are like those of early lactation. It may be argued that the combined treatment resulted only in additive, rather than potentiative changes in some constituents. Nonetheless, the relative changes in nucleic acid concentrations brought about by the combination, about two-fold for DNA, and two of three-fold for RNA, are similar to those reported by Kirkham and Turner (2) for total nucleic acids. Experimentally-developed glands show succinoxidase and cytochrome oxidase activities similar to those of glands from pregnant rats.

The increase in arginase per unit DNA was the clearest biochemical in-

TABLE 4. ARGINASE OF EXPERIMENTALLY-DEVELOPED GLANDS

Treatment	Number of Groups	Units per mg.:*		
		FW	TN	DNA
I. Vehicle	4	0.7±0.2	98±48	173±70
II. Progesterone	4	0.7±0.2	70±7	115±38
Prob. of diff. II→IV		<0.001	<0.001	<0.001
III. Estradiol	7	0.4±0.1	41±8	79±16
Prob. of diff. III→IV		<0.001	<0.001	<0.001
IV. Estradiol and Progesterone	7	4.9±1.1	389±62	595±38

* Values are μM urea formed/minute/l. reacting solution/mg.

dication of synergism of estrogen and progesterone on the mammary glands. Its appearance significantly correlated with the development of the lobulo-alveolar system. Unfortunately, histochemical methods for arginase localization are not available, for it would be of interest to know if most of the activity were located in the lobules. The relatively high activity in mammary tumors (17), comprised chiefly of acinar elements, suggests this may be the case.

Folley and Greenbaum (18) observed no significant elevation in mammary arginase activity during pregnancy, when the gland is presumably under the influence of estrogen and progesterone stimulation, but noted a later marked rise during mid to late lactation. The dramatic increase in arginase induced by this steroid combination was therefore somewhat surprising and may be due to a difference in effective dosage. Direct comparison of absolute enzyme activities in the two studies is precluded by different assay methods. When comparison is made to the activities of glands from pregnant or lactating rats in our own laboratory, activity of the artificially-stimulated glands is considerably less.

The mammary glands of small laboratory animals, developed in this manner by a synergistic steroid combination, should provide a suitable standardized endpoint for assay of lactation-stimulating drugs. Since both succinoxidase and cytochrome oxidase activities show a prompt rise at parturition (16), changes in these enzymes should provide a good basis for assay of lactogenic (lactation-initiating) potency of hormones and drugs in animals suitably prepared with steroids. Similarly, arginase activity, which increases during lactation (18), should provide a valid measure of lactopoetic (lactation-maintaining) potency.

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EFFECT OF DI-ETHYL ETHER ANESTHESIA ON THYROID FUNCTION OF RATS

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ABSTRACT

The mechanism of the action of ether anesthesia on thyroid function was studied. Ether anesthesia did not significantly influence the following: (1) radioactive iodine (I^{131}) absorption from the peritoneal cavity, (2) gastrointestinal tract or urinary excretion of I^{131}, (3) serum concentration of I^{131}, or (4) T/S ratio. The uptake and secretion of radioiodine by rat thyroid was significantly inhibited by administration of ether anesthesia. Thiopental, however, if anything, increased the release of radioiodine from the thyroid. Pentobarbital (Nembutal) had no effect.

Radioactive protein-bound iodine (PBI^{131}) of the rat thyroid was significantly less in the ether anesthetized groups than in the control animals. Paper chromatographic study of the thyroid showed that in the ether anesthetized groups, in contrast with the control rats, the content of thyroxine plus triiodothyronine was markedly reduced compared to the reduction in the content of moniodotyrosine plus diiodotyrosine.

ANESTHETIC agents containing a thiocarbonamide structure, such as thiopental and thiamylal, have been reported to have anti-thyroid activity (1, 2). Recently di-ethyl ether anesthesia has been shown to depress thyroid function as indicated by its effect on the uptake of radioiodine by the thyroid (3).

The present work was undertaken in an attempt to clarify the mechanism of action of ether anesthesia on the thyroid. It includes the quantification of the following: (1) the gastrointestinal distribution of radioiodine (I^{131}), (2) absorption of I^{131} from peritoneal cavity, (3) renal excretion and serum concentration of radioiodine, (4) thyroid: serum iodide concentration ratio (T/S), (5) secretion of radioiodine from thyroid, (6) the thyroidal protein-bound radioiodine, and (7) the relative amounts of the thyroidal iodinated compounds.

MATERIALS AND METHODS

Determination of radioiodine in the gastrointestinal tract, peritoneal cavity, renal excretion and serum concentration

Sprague-Dawley rats weighing 100–180 gm. were fed Remington's low iodine diet for a week prior to the experiments. After 2 minutes of di-ethyl ether anesthesia, 1 μc. of I^{131} was injected intraperitoneal as 0.5 ml. solution. These rats were kept under

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ether anesthesia for 2 hours, sacrificed at the end of anesthesia, and the thyroids were removed and blood samples were obtained from the heart. The urine was collected in a glass flask from a metabolism cage and from the bladder by puncture. The radioactivity of the thyroid, serum, urine, peritoneal washings and various organs was determined by using a well-type scintillation counter as described earlier (3).

Determination of T/S ratio

The thyroid: serum iodide concentration ratio (T/S) was determined in some animals according to the method of Vanderlaan and Greer (1950). The rats were fed propylthiouracil (PTU) 0.03% concentration in Remington's diet for 3 days prior to experiments. The animals received 5 mg. of PTU subcutaneously and 30 minutes later ether anesthesia was administered for 2 hours. The I^{131} (1 μc) was injected 2 minutes after anesthesia started and all animals were sacrificed at the end of anesthesia. The T/S ratio was expressed at the calculated radioactivity of 100 mg. thyroid tissue divided by the activity of the serum sample of 0.1 ml.

Secretion of radioiodine

Other animals, 40 hours after an intraperitoneal injection of 5 μc of I^{131}, were injected with 10 mg. of thiouracil as 1 ml. of a slightly alkaline solution, and were fed Remington's diet with 0.03% of thiouracil. Di-ethyl ether anesthesia was administered for 2 hours *46* hours after I^{131} injection ("zero time"). Thiopental and pentobarbital (Nembutal) were given to other animals at "zero time" (40 mg./kg. intraperitoneally). The thyroid region of the rats was counted while under light ether anesthesia for about 2 minutes.

Measurement of radioactive protein-bound iodine (PBI-131)

Radioactive protein-bound iodine (PBI^{131}) of the thyroid glands was determined by the following method: the dissected thyroid glands were homogenized with 1 ml. of 10% trichloroacetic acid solution. After centrifugation the supernatant was discarded and the precipitate (PBI^{131}) was then washed with 1 ml. of 10% trichloroacetic acid solution. This procedure was repeated three times, and the radioactivity of the precipitate was determined in a well type scintillation counter.

Chromatographic analysis of thyroid tissues

For the paper chromatographic study, the thyroids were homogenized and hydrolysed with pancreatin for 24 hours at 37° C in Krebs bicarbonate at pH 7.4 with propylthiouracil 0.003%. The hydrolysate was chromatographed in butanol-acetic acid-water (75:10:15) and butanol-ethanol-2*N* NH_4OH (5:1:2) ascending system, radioautographed, and the radioactivity was counted by a strip counter.

RESULTS

1. *The distribution of radioiodine in gastrointestinal tract*

Administration of di-ethyl ether anesthesia for 2 hours caused no significant alteration in accumulation of I^{131} in gastrointestinal tract (Table 1). The control group had 24.3% and the ether group 26.1% of the injected I^{131} in the gastrointestinal tract. The stomach had the highest amount of I^{131} in both groups, but the difference between the two groups was not statistically significant (Table 1). Considerable I^{131} was also found in the small intestine, liver and the large intestine. There was no inhibition by ether anesthesia of absorption of the I^{131} from the peritoneal cavity.

TABLE 1. EFFECT OF DI-ETHYL ETHER ANESTHESIA FOR 2 HOURS ON DISTRIBUTION OF I^{131} IN VARIOUS ORGANS IN RATS

	Control	Ether	
No. of rats	9	8	
Body weight (gm.)	116 ± 4*	119 ± 3	
Thyroid	10.6 ± 1.1	6.5 ± 0.9	t = 2.4, P < 0.05
Large intestine	1.8 ± 0.4	2.5 ± 0.7	
Small intestine	5.3 ± 0.5	4.5 ± 0.3	
Liver	4.1 ± 0.4	3.1 ± 0.3	
Stomach	12.6 ± 0.9	15.7 ± 1.9	t = 1.4, P < 0.10
Peritoneal cavity	0.02	0.02	
Pancreas	0.4	.3	
Kidney	1.0 ± 0.05	0.9 ± 0.15	
Urine volume (cc.)	3.4	2.3	
$\%I^{131}$	2.3	2.4	
Heart	0.2	0.2	
Serum (/cc.)	0.5 ± 0.09	0.7 ± 0.04	t = 0.8, P < 0.50

* Mean ± standard error.
Figures are expressed as % of the administered I^{131}.

TABLE 2. EFFECT OF DI-ETHYL ETHER ANESTHESIA FOR 2 HOURS ON THE THYROID: SERUM IODIDE CONCENTRATION (T/S) RATIOS IN NORMAL RATS

Group	No. rats	Body weight gm.	Thyroid weight mg.	Thyroid : serum radio-iodide gradient
Control	7	119 ± 4*	14 ± 1	73.9 ± 6.0
Ether	7	106 ± 2	13 ± 1	64.6 ± 9.0

* Mean ± standard errors.

2. *Renal excretion and serum concentration of I^{131}*

Renal excretion of I^{131} was similar in ether anesthesia (2.4%) and control groups (2.3%), despite the fact that the urine volume of the anesthetized group (2.3 ml.) was less than that of the control animals (3.3 ml.). Serum

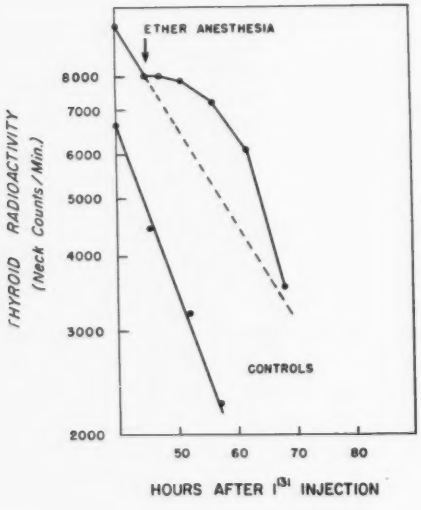

FIG. 1. Effect of Di-ethyl Ether Anesthesia on Thyroidal Release of I^{131} in Rats. Twelve rats were in each group. These animals were given thiouracil at 40 hours after I^{131} injection.

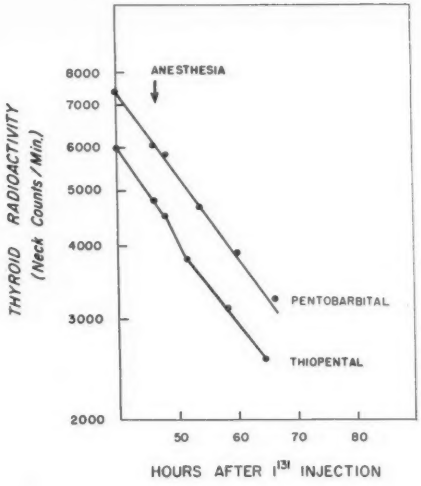

FIG. 2. Effect of Thiopental and Pentobarbital (Nembutal) on Thyroidal Release of I^{131} in Rats. Nine rats were in each group.

concentration of radioiodine in the ether group (0.7%) was not significantly higher than in the control group (0.5%).

3. *Thyroid: Serum iodide concentration ratio (T/S)*

Ether anesthesia did not significantly influence (87% of the control) the T/S ratio as shown in Table 2 ($t=0.8$, $P<0.40$).

4. *Thyroid uptake and secretion of I^{131}*

Di-ethyl ether anesthesia significantly depressed thyroidal uptake of I^{131} (Table 1). After the correction for the physical decay of the I^{131}, the fall in thyroid I^{131} content while on thiouracil occurred exponentially (Figs. 1 and 2). Radioiodine remaining in the thyroid of the ether anesthetized animals at 4-hours post anesthesia was 94.4% in contrast with 75.9% in the control animals (Table 3). At 10-hours post anesthesia I^{131} remaining in the thyroid of the ether groups was still significantly higher than in the control group. A prominent slowing in the rate of release in thyroidal radioiodine was observed during ether anesthesia and post anesthesia (Fig. 1). In contrast,

TABLE 3. EFFECT OF DI-ETHYL ETHER ANESTHESIA ON THYROIDAL I^{131} SECRETION IN RATS

Interval after "zero time"*	Control	Ether
	I^{131} remaining in thyroid	I^{131} remaining in thyroid
0	100	100
6 hours	75.9 ± 2.3†	94.4 ± 1.5 ($t=7.0$, $P<0.001$)
12 hours	60.1 ± 2.9	82.0 ± 3.2 ($t=2.8$, $P<0.01$)

* The "zero time" count was made 46 hours after I^{131} injection and 6 hours after thiouracil. Anesthesia started from "zero time" for 2 hours, 12 rats were in each group.
† Mean ± standard error.

TABLE 4. EFFECT OF DI-ETHYL ETHER ANESTHESIA ON 2 HOURS THYROID UPTAKE OF I^{131} AND THYROIDAL PBI^{131}

	No. of rats	Thyroid uptake % of administered I^{131}	PBI^{131} % of thyroidal I^{131}
Control	9	9.5±1.1*	75.1±3.2
Ether	7	5.1±1.0**	57.5±3.4†

* Mean ± standard error
** $t = 2.7$, $P < 0.02$.
† $t = 3.2$, $P < 0.01$.

thiopental and Nembutal had no similar effect on the release of thyroidal radioiodine (Fig. 2).

5. *Thyroidal PBI-131 and the relative amounts of the thyroidal iodinated compounds*

Thyroid protein-bound radioiodine (PBI^{131}) was significantly less in ether anesthetized animals (57.5%) than in the control rats (75.1%) (Table

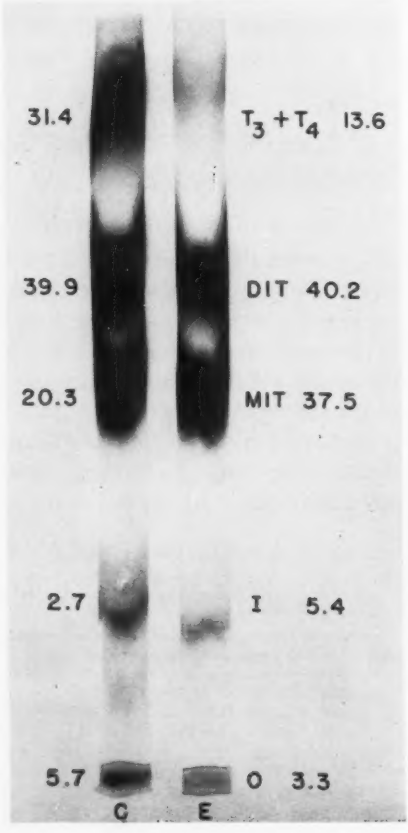

FIG. 3. Radioautograph of filter paper chromatogram showing the distribution of I^{131} in pancreatin digests of pooled thyroids 2 hours after I^{131} (20 μc) injection. Ether anesthesia was given for 2 hours and each group consisted of 5 rats. The following abbreviations were used: C, control group; E, ether group; O, origin; I, inorganic iodide; MIT, monoidotyrosine; DIT, diiodotyrosine; T_3, triiodothyronine; T_4, thyroxine. The numbers indicate the percentage of the I^{131} on the chromatogram present in the various components. Solvent, butanol-acetic acid-water.

4). Chromatography of thyroidal hydrolysates in 2 experiments indicated a marked diminution in thyroxine content and an increase in the ratio of mono- to diiodotyrosine in the ether anesthetized animals (1:1.1) as compared to the control rats (1:1.9) (Fig. 3).

DISCUSSION

The amount of I^{131} accumulated by the thyroid is proportional to the concentration of I^{131} in the blood. If thyroid activity remains at a constant level and its vascular supply is unchanged, the amount of I^{131} collected by the thyroid will be determined by factors which influence its concentration in the blood. The ability of the kidney to excrete iodide is the main factor modifying the blood concentration of I^{131}. In the first hours after the injection of I^{131}, however, the gastrointestinal and salivary concentration of iodine may be important. Brown (4) reported that 70% of the injected dose of radioiodine was secreted as iodide by the salivary and gastric glands, one-half of the total in the first 4 hours, and the salivary glands contributed about one-fifth of this. In the present study, the depressed thyroidal collection of radioiodine caused by ether anesthesia was not due to an increased gastrointestinal accumulation of I^{131} from the peritoneal space (Table 1).

Urinary excretion of I^{131} was not significantly influenced by ether anesthesia. In addition the findings confirm the observation by Bricker and Hlad (5) who reported that urinary excretion of I^{131} is independent of the urine volume. The depression of thyroidal uptake of I^{131} by ether anesthesia was not due to an increased urinary output of radioiodine (Table 1). The remainder of the I^{131} (about 55%) was presumably distributed throughout the body in the extracelluar fluid space. T/S ratio of I^{131} was not significantly depressed by ether anesthesia, which indicates that the thyroidal concentrating mechanism for iodide was not influenced by the ether anesthesia (Table 2).

The present study confirmed the previous reports (6–9) that the rate of the thyroidal I^{131} release from the blocked and unblocked gland follows an exponential curve. Di-ethyl ether anesthesia clearly demonstrated an inhibition of the release of I^{131} from the rat thyroid blocked with thiouracil which lasted for at least 12 hours. Thiopental, however, did not inhibit but possibly slightly increased the release of thyroidal I^{131} in the post anesthesia period (Figs. 1 and 2). This difference was not accounted for by the sleeping time or the length of inactivity in the post anesthesia period. The rats slept from 10 to 15 minutes after ether anesthesia was discontinued and regained full preanesthetic activity 20 to 30 minutes later. Thiopental and Nembutal groups slept approximately for 2 hours, and resumed preanesthetic activity three to three and a half hours later.

Administration of thiopental resulted in a more prolonged and severe depression of thyroidal accumulation of I^{131} than ether (3). These findings indicate that the influence of di-ethyl ether anesthesia on rat thyroid is

different from that of thiopental on both thyroidal uptake and release of I^{131}. The difference in the effect on uptake was a quantitative one and could be due to rapid excretion of ether as opposed to slow excretion of thiopental. The difference in the effect on release of I^{131} was a qualitative one as the release rate of I^{131} from the thiouracil blocked gland was markedly slowed by ether anesthesia.

The action of thiopental was postulated by Wase and Foster (2) to be due to the breakdown of thiopental to thiourea in the body. Therefore, thiopental in effect, may have acted directly on the thyroid gland as an anti-thyroid compound. Interestingly, pentobarbital seems to have no

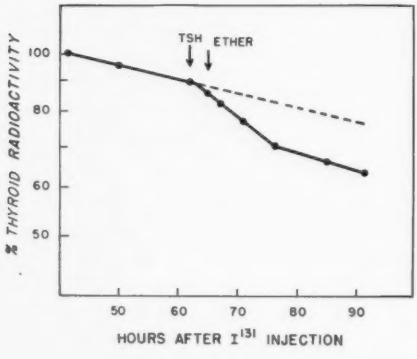

FIG. 4. Effect of Di-ethyl Ether Anesthesia on Thyroidal Release of I^{131} in Hypophysectomized Rats. Note: Acceleration of TSH of thyroidal release of I^{131} was not blocked by ether anesthesia. The effect of TSH lasted for 10 hours. In another experiment the effect of TSH alone and with ether in hypophysectomized rats on the thyroidal release rate of I^{131} was almost identical. TSH (2.8 U.S.P. unit of U.S.P. reference standard, subcutaneous single injection) was given at 64 hours after 15 μc. of I^{131} was injected. Fifteen rats, one week after operation, were in each group. Thiouracil treatment was started 40 hours after I^{131} injection as in Figs. 1 and 2. Ether anesthesia, for 2 hours, was started 3 hours after TSH injection.

significant effect on thyroid release of I^{131} confirming the report of Perry (8). Pentobarbital has no SH group in its chemical structure and Wase *et al.* (10) reported that it did not reduce I^{131} uptake by rat thyroid.

It is of interest to note that ether anesthesia did not inhibit the acceleration of I^{131} release caused by a large dose of exogenous thyrotropic hormone (TSH) in hypophysectomized rats (Fig. 4). This would suggest that ether interferes with release mechanisms, independent of any effect of TSH. This does not, however, prove that the effect of ether is not inhibiting through endogenous TSH in the intact animal.

Protein-bound radioiodine formation in the thyroid was significantly depressed by ether anesthesia (Table 4). The analysis of the various iodinated compounds in the thyroid by paper chromatography indicated, in 2 experiments, an increase in the ratio of monoiodotyrosine to diiodotyrosine and a reduction in the amount of thyroxine in the rats given ether anesthesia. In 2 additional experiments, however, a change in the ratio of monoidotyrosine to diiodotyrosine could not be clearly determined, but

there was a definite diminution in the amount of thyroxine and triiodothyronine relative to the decrease in the amount of monoiodotyrosine and diiodotyrosine in the ether groups.

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HORMONAL INFLUENCES ON THE CARBONIC ANHYDRASE CONCENTRATION IN THE ACCESSORY REPRODUCTIVE TRACTS OF THE RAT[1]

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ABSTRACT

The effects of sex hormones on the carbonic anhydrase activity of the rat accessory organs of reproduction were studied.

Ovariectomy causes a marked increase in enzyme concentration, but does not change the total enzyme content of the uteri, indicating that the normal amount of the enzyme was maintained in uteri atrophied by ovariectomy.

Estradiol produces marked decreases of the uterine enzyme concentration of ovariectomized rats, the extent of which depends upon the dosage.

When progesterone is injected along with estradiol, it partially inhibits the effect of estradiol on the uterine enzyme of spayed rats, but progesterone by itself has no effect at all on the concentration of uterine enzyme.

Eight and fifteen days after castration, the enzyme concentration is markedly increased in the ventral prostates of adult rats but is not changed in the seminal vesicles; sixty-five days after castration, the concentration is decreased in both tissues. Also, in immature rats, castration produces in 10 days a marked increase of the enzyme concentration in the ventral prostates but no change of enzyme concentration in the seminal vesicles.

Testosterone injected into immature castrated rats increases the enzyme concentration of the seminal vesicles and decreases that of the ventral prostates.

Estradiol in immature normal rats decreases the enzyme concentration of the seminal vesicles and increases that of the ventral prostates. When administered with testosterone, estradiol enhances the effect of testosterone on tissue weights, but does not modify the effect of testosterone on the enzyme concentrations.

A large dosage of progesterone (30 mg. per rat) has a slight ability to increase the enzyme concentrations of both tissues of immature male rats.

Cortisone in a total dose per rat of 1.2 mg. has no significant effect on the enzyme activity in either tissue.

The above results suggest that the carbonic anhydrase concentrations in the uteri, seminal vesicles and ventral prostates of rats are under the control of sex hormones.

IT HAS been demonstrated that the endometrial carbonic anhydrase content of rabbit uteri is under the control of progestin and estrogen (1, 2, 3, 4). However, negative results were obtained by Lutwak-Mann

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(1) in attempts to estimate the carbonic anhydrase content of rat uteri and to stimulate the uterine enzyme activity by injecting progesterone or gonadotropin. Few experiments have been carried out on the hormonal influences on the carbonic anhydrase content in the accessory reproductive tracts of male rats, although the enzyme and zinc content in the prostate glands of rat and rabbit were investigated by Mawson *et al.* (5). Using the more sensitive method of enzyme determination described in our previous paper (6), the effects of sex hormones on the carbonic anhydrase content of the rat accessory tracts of reproduction have been studied.

In this paper, we present: (a) the effects of ovariectomy, progesterone, and estradiol with and without progesterone on the uterine carbonic anhydrase of adult rats; (b) the effects of castration on the enzyme concentrations in the seminal vesicles and ventral prostates of adult and immature rats; (c) the effects of testosterone on the enzyme concentrations in the seminal vesicles and ventral prostates of immature castrated rats; and (d) the effects of various hormones—cortisone, progesterone, estradiol and testosterone with and without estradiol—on the enzyme concentrations in the seminal vesicles and ventral prostates of immature normal rats.

METHODS

Male and female Sprague-Dawley albino rats were used. Most hormones were dissolved in Sesame Oil U.S.P. The ovariectomized adult females were injected subcutaneously in the neck with 0.1 to 0.2 ml. once daily for 6 days; the normal and castrated immature male rats for 10 days. Injections were started on the day of operation. The animals were killed with ether 24 hours after the last injection.

To make each enzyme extract, a group of animals was used; 3 to 4 rats for a uterine extract and 3 to 5 rats for an extract of seminal vesicles or ventral prostates. The uteri, seminal vesicles and ventral prostates were isolated. The uteri and seminal vesicles were opened longitudinally. All tissues were rinsed with cold saline before weighing. The groups of tissues were put into Ten Brock homogenizers, chopped into small pieces with scissors, and homogenized with a ten to twenty-fold volume of cold distilled water. The homogenates were transferred into 15 ml. graduated centrifuge tubes and centrifuged at 1500 r.p.m. for ten minutes. After recording the volumes, the supernatant fluid was decanted and used for the determination of enzyme activity. The procedure of enzyme determination was the same as that described in our previous paper (6). The enzyme unit per gram wet tissue (EU/g) was calculated as follows:

$$\mathrm{EU/g} = \frac{1000}{\mathrm{CV}},$$

C: Concentration of extract (mg./ml.)
V: Volume of extract which is required to shorten the reaction time from 65–75 (control) to 25–30 seconds.

RESULTS

Effect of ovariectomy on uterine carbonic anhydrase

Adult female rats weighing about 160 gm. were used. One half were ovariectomized, the other half were left intact. Seven days after the day of

TABLE 1. EFFECT OF OVARIECTOMY ON THE UTERINE CARBONIC ANHYDRASE ACTIVITY OF ADULT RATS

Group	No. of Rats	Average Body Weight (gm.)	Average Uterine Weight (mg.)	Carbonic Anhydrase Activity of Uterus	
				EU/gm. Uterus	EU/whole Uterus
Normal Control	3	196	248	5.7	1.414
	3	194	263	6.3	1.657
	3	180	275	8.6	2.365
	3	177	318	7.4	2.353
	3	192	312	7.1	2.215
	3	192	270	9.3	2.511
	3	189	317	7.0	2.219
	Mean	189	286	7.3 ± 0.5*	2.105 ± 0.154*
Ovariectomized (7 days after operation)	3	198	124	21.4	2.654
	3	198	104	16.0	1.664
	3	182	118	20.0	2.360
	3	186	118	23.4	2.761
	3	197	103	20.1	2.070
	3	203	122	19.5	2.379
	3	194	94	22.0	2.068
	Mean	194	112	20.3 ± 0.9*	2.274 ± 0.142*

EU/whole uterus: EU/gm. uterus × average uterine weight (gm.)
* Mean ± S.E.

operation, both groups of animals were sacrificed, the uterine weights recorded and the enzyme activity of the uterine extracts measured. The results are shown in Table 1. Ovariectomy caused marked decreases of the uterine weights, with significant increases in enzyme concentration (EU/g uterus). However, the total enzyme contents in the uteri (EU/whole uterus) were not significantly changed by ovariectomy.

Effects of estradiol and progesterone on uterine carbonic anhydrase

Various dosages of estradiol or progesterone were injected subcutaneously once daily for 6 days into ovariectomized adult rats. The uterine

TABLE 2. EFFECTS OF ESTRADIOL AND PROGESTERONE ON THE UTERINE CARBONIC ANHYDRASE ACTIVITY OF OVARIECTOMIZED ADULT RATS

Hormone & Dosage		No. of Rats	Average Body Weight (gm.)	Average Uterine Weight (mg.)	No. of Uterine Extracts	EU/g Uterus (Mean ± S.E.)
Control (Sesame oil)		62	171	120	16	24.7 ± 0.9
Estradiol	0.3 μg.	29	167	201	8	23.6 ± 1.4
	0.6 μg.	18	163	316	6	14.7 ± 1.8
	1.2 μg.	23	158	284	8	7.5 ± 0.6
	3.0 μg.	9	165	296	3	<6.3
Progesterone	0.03 mg.	12	174	120	4	28.0 ± 1.7
	0.06 mg.	12	176	132	4	27.4 ± 1.4
	0.12 mg.	16	168	106	4	24.4 ± 1.3
	0.6 mg.	20	170	125	6	23.2 ± 1.9
	3.0 mg.	17	179	138	5	22.9 ± 0.8

TABLE 3. ANTAGONISTIC EFFECT OF PROGESTERONE ON THE DECREASE IN RAT UTERINE CARBONIC ANHYDRASE ACTIVITY PRODUCED BY ESTRADIOL

Dose of Estradiol (μg.)	0	1.2	1.2	1.2	1.2	1.2
Dose of Progesterone (mg.)	0	0	0.12	0.6	3.0	6.0
Carbonic Anhydrase Activity of Uteri (EU/gm. wet tissue)	23.7	6.5	6.0	9.3	11.4	6.9
	23.8	6.6	7.0	6.7	17.2	10.4
	23.2	7.2	5.8	5.4	15.1	8.0
	21.7	5.3	6.6	8.2	8.8	9.1
	30.0	6.5	6.5	8.0	6.5	14.3
	16.2	4.0		11.4	9.3	11.0
	18.8	4.8			6.3	
	22.1					
	18.7					
Mean	22.0	5.8	6.4	8.2	10.7	10.0

weights and the enzyme contents of the hormone-treated group were compared with those of the control group. The data shown in Table 2 demonstrate that progesterone over the dosage range 0.03 to 3 mg. had no effect on the uterine weights or the enzyme concentration, whereas estradiol over the dosage range 0.3 to 3 μg. produced marked decreases in the enzyme concentration, the extent of which depended upon the dosage. The decreases of the uterine enzyme concentration by estradiol were not inversely proportionate to the increases of the uterine weights, indicating not only dilution of the enzyme with the hypertrophy of uterus but a decrease in the biosynthesis of uterine carbonic anhydrase.

Effects of estradiol-progesterone combinations on the uterine carbonic anhydrase

A series of dosages of progesterone with and without 1.2 μg. of estradiol were injected subcutaneously for 6 days into ovariectomized rats to investigate the effect of progesterone on the decreased uterine carbonic anhydrase concentration produced by the activity of estradiol. The data presented in Table 3 demonstrate that progesterone produced partial inhibition of the estradiol effect, the extent of which depended upon the dosage. However, complete inhibition of the effect of 1.2 μg of estradiol was not obtained by progesterone at dosages up to 6 mg. There were no significant differences in the uterine weights of the estradiol-treated animals either with or without progesterone.

Effects of castration on the carbonic anhydrase of seminal vesicles and ventral prostates of adult rats

Castrated adult male rats weighing about 300 grams were killed along with an intact control group 8, 15 and 65 days after castration. The average weights and the carbonic anhydrase contents of the seminal vesicles and ventral prostates were measured. The results are shown in Table 4. Both tissues showed reductions in weight and size according to the period

TABLE 4. THE EFFECT OF CASTRATION ON THE CARBONIC ANHYDRASE ACTIVITY OF THE SEMINAL VESICLES AND VENTRAL PROSTATES OF ADULT RATS

Days After Castration	No. of Rats	Average Body Weight (gm.)	Seminal Vesicles		Ventral Prostate	
			Average Weight (mg.)	EU/gm. Wet Tissue	Average Weight (mg.)	EU/gm. Wet Tissue
Normal	24	339	403	442	445	101
8	12	299	133	407	55	258
15	15	305	115	364	50	359
65	8	317	28	183	12	91

after castration. Up to 15 days after castration, no significant difference was observed in the enzyme concentration of the seminal vesicles of the normal and castrated animals; however, marked increases of the enzyme concentration occurred in the ventral prostates of the castrated groups. On the 65th day after castration, when both tissues were atrophied to the level of immature rats, the enzyme contents of both tissues were reduced.

Effects of castration and testosterone on the carbonic anhydrase of seminal vesicles and ventral prostates of immature rats

In Table 5, we present the data on the tissue weights and the enzyme activities of normal, castrated and testosterone-injected castrated imma-

TABLE 5. THE EFFECT OF CASTRATION AND TESTOSTERONE ON THE CARBONIC ANHYDRASE ACTIVITY OF THE SEMINAL VESICLES AND VENTRAL PROSTATES OF IMMATURE RATS

Treatment	No. of Rats	Average Body Weight (gm.)	Seminal Vesicles		Ventral Prostate	
			Average Weight (mg.)	EU/gm. Wet Tissue	Average Weight (mg.)	EU/gm. Wet Tissue
Normal (Sesame Oil)	4	113	21.8	254	62.0	34
	4	121	28.4	295	72.8	87
	4	90	16.2	334	37.3	44
	4	106	23.9	313	59.6	41
	4	105	19.5	340	52.1	44
	Mean	107	22.0	307 ± 15.5*	56.8	50 ± 2.2*
Castration + Sesame Oil	4	91	7.0	357	14.0	227
	4	86	7.2	379	11.3	111
	4	86	7.5	291	9.0	208
	4	91	7.2	247	10.0	238
	3	84	6.3	280	8.8	286
	Mean	88	7.0	311 ± 24.7*	10.6	214 ± 28.8*
Castration + Testosterone 2.4 mg.	4	95	93	676	109	32
	4	84	100	649	109	124
	4	85	96	490	98	62
	4	93	100	610	114	52
	4	87	106	562	122	105
	Mean	89	99	597 ± 33.0*	110.4	75 ± 17.1*

* Mean ± S.E.

ture animals. The testosterone was dissolved in sesame oil and injected subcutaneously once daily for 10 days. The data demonstrated that castration produced a marked decrease in the weights of both tissues, no change in the enzyme concentration of the seminal vesicles, but an apparent increase in the enzyme concentration of the ventral prostates. This corresponded to the data obtained using adult rats. When testosterone was administered to the castrated animals, the enzyme concentration increased in the seminal vesicles but decreased in the ventral prostates in spite of the increase in their weights.

In Table 6, we present the data on the enzyme activities of ventral prostates and seminal vesicles in a series of control and testosterone-injected castrated immature rats. Testosterone was injected subcutaneously once daily for 10 days as a fine crystal suspension made with Suspending Vehicle Formula #17874, which consists of an aqueous solution of sodium chloride (0.9%), polysorbate 80 (0.4%), carboxy methyl cellulose (0.5%) and benzyl alcohol (0.9%). The data demonstrate that testosterone produced decreases of the enzyme concentration in the ventral prostates and increases of the concentration in the seminal vesicles, the extents of which depended upon the dosage.

Effects of typical steroidal hormones on the carbonic anhydrase of seminal vesicles and ventral prostates of immature rats

The hormones—cortisone, testosterone with and without estradiol, progesterone and estradiol—were injected subcutaneously once daily for 10 days into 22-day old male rats to investigate the effect on the carbonic anhydrase concentration of the seminal vesicles and ventral prostates. The results are presented in Table 7. Cortisone (1.2 mg.) had no effect on the weights and enzyme activities of either tissue. A large amount of

TABLE 6. EFFECTS OF TESTOSTERONE ON THE CARBONIC ANHYDRASE ACTIVITY OF THE VENTRAL PROSTATES AND SEMINAL VESICLES OF CASTRATED IMMATURE RATS

Carbonic Anhydrase Activity (EU/gm. Tissue)	Total Dose of Testosterone (mg.)		0	0.3	0.6	1.2	2.4
	Ventral Prostate	A	—	258	167	169	233
		B	150	195	258	165	155
		C	335	145	286	—	122
		D	287	354	116	190	179
		E	190	—	185	—	167
		F	258	—	130	—	132
		Mean	244	238	190	175	165
	Seminal Vesicles	A	—	362	244	417	435
		B	189	275	351	481	371
		C	191	178	787	196	455
		D	388	290	311	315	521
		E	253	—	400	—	431
		F	291	—	317	—	385
		Mean	262	276	402	352	433

TABLE 7. THE EFFECTS OF STEROIDAL HORMONES ON THE CARBONIC ANHYDRASE ACTIVITY OF SEMINAL VESICLES AND VENTRAL PROSTATE OF IMMATURE RATS

Treatment	No. of Rats	No. of Extracts	Average Body Weight (gm.)	Seminal Vesicles		Ventral Prostate	
				Average Weight (mg.)	EU/gm. Wet Tissue	Average Weight (mg.)	EU/gm. Wet Tissue
Control (Sesame Oil)	46	13	92	14	237 ± 8.8*	50	84 ± 12.5*
Cortisone 1.2 mg.	19	4	89	15	222 ± 8.0	53	84 ± 21.7
Progesterone 30 mg.	15	5	97	17	306 ± 41.3	69	279 ± 65.0
Estradiol 0.5 μg.	20	5	91	14	225 ± 16.3	41	138 ± 1.5
Estradiol 5 μg.	20	5	85	17	172 ± 19.5	26	202 ± 16.6
Estradiol 50 μg.	15	5	83	26	53 ± 10.3	14	233 ± 14.5
Testosterone 1.2 mg.	14	5	89	80	431 ± 15.9	89	142 ± 10.8
Testosterone 2.4 mg.	12	4	105	95	536 ± 4.0	114	134 ± 33.6
Testosterone 2.4 mg. + Estradiol 5 μg.	12	3	91	113	461 ± 24.6	118	129 ± 4.6
Testosterone 2.4 mg. + Estradiol 50 μg.	12	4	101	120	528 ± 29.2	121	103 ± 29.7

* Mean ± S.E.

progesterone (30 mg.) appeared to increase slightly the enzyme concentrations of both tissues although the mean responses were accompanied by a wide range of standard error. Estradiol produced an increase of weight and a decrease of the enzyme concentration in the seminal vesicles, but it produced the reverse reaction in the ventral prostates. Testosterone increased the enzyme activity markedly in the seminal vesicles but only slightly in the ventral prostates. When estradiol was injected along with testosterone, it enhanced slightly the effect of testosterone on the tissue weights but did not modify the effect on the enzyme concentration.

DISCUSSION

In our previous experiments with Clauberg rabbits (4), the inhibition of progesterone by estrogens was demonstrated by endometrial carbonic anhydrase titers correlated with the degrees of progestational proliferation as measured by the G/M ratio (glandular/total muscosal area). Also the complete inactivity of estrogens by themselves was shown in both measurements. In experiments with rats however, Lutwak-Mann (1) obtained negative results in attempts to estimate the carbonic anhydrase content of uteri and to stimulate the enzyme activity by injecting progesterone or gonadotropin. By making each uterine enzyme extract from a group of three or more rats and using the more sensitive method of enzyme determination described in our previous paper (6), it has been possible to estimate the enzyme concentration in the non-pregnant uteri of rats, both normal and ovariectomized. Our data demonstrate that ovariectomy causes a marked increase of the uterine carbonic anhydrase concentration without

changing the total amount of enzyme in the uteri, indication that the normal amount of the enzyme remains in the uteri atrophied by ovariectomy. Administration of estradiol prevents the increase in the uterine enzyme concentration resulting from ovariectomy. Progesterone partially inhibits the effect of estradiol on the uterine enzyme concentration in ovariectomized rats in spite of the complete inactivity of progesterone by itself. This suggests an indirect effect of progesterone on the estrogen-stimulated processes e.g. an alteration of estradiol metabolism within the uterine tissue, or a counteraction of the estrogen-stimulated synthesis of proteins other than carbonic anhydrase. In any event, the uterine carbonic anhydrase concentrations of rats and rabbits are under the control of progesterone and estrogen, the progesterone taking the initiative in rabbits and the estrogen in rats.

The advantages of a chemical approach to the study of the physiological function of male accessory organs of reproduction have been obvious, as reviewed by Mann and Lutwak-Mann (7, 8). However, few experiments have been carried out on the hormonal influences on the carbonic anhydrase contents in the accessory genital tracts of male rats, although the enzyme and zinc content in the prostate glands of the rat and rabbit were investigated by Mawson and Fisher (5). We have studied the concentration of carbonic anhydrase in rat seminal vesicles and ventral prostates, the weights of which have ordinarily been used as the criteria of the biological activity of androgenic hormones. The data demonstrate that castration produces, in 8, 10, and 15 days, a marked increase of the enzyme concentration in the ventral prostates of adult and immature rats, whereas no change of the enzyme concentration was observed in the seminal vesicles. Testosterone injected into immature castrated rats increases the enzyme concentration of the seminal vesicles, and decreases that of the ventral prostates. Consequently, in spite of the well-known fact that the secretory output and the weights of both the seminal vesicles and the ventral prostates are strictly regulated in the same direction by the internal secretion of the testes, the influence of castration and testosterone treatment on the carbonic anhydrase concentration in the rat ventral prostates is entirely opposite to the influence on the enzyme in the seminal vesicles. This discrepancy should be clarified by further investigation. However, Pincus (9) has mentioned that the synthesis of additional tissue enzyme is not based on the general protein anabolic activity of sexagens on tissues but may result from the redistribution of tissue nitrogen. The lack of relationship between the effect of sex hormones on the carbonic anhydrase concentrations and the effect on the tissue weights agrees with this explanation. Thus testosterone-stimulated protein synthesis in the seminal vesicles involves increased production of carbonic anhydrase, whereas in the ventral prostate a shunt into the synthesis of other proteins appears to occur.

Among the chemical peculiarities of the prostate gland is its rather high content of zinc, which is a metallic component of carbonic anhydrase. However, the use of zinc levels or radioactive zinc uptake for estimating the carbonic anhydrase content or for supporting measurements of the enzyme activity is not valid in biological material other than blood, since zinc exists in tissues in forms other than or in addition to carbonic anhydrase, as emphasized by Vallee and Altschule (10). Mawson and Fisher (5) noticed that the zinc content and carbonic anhydrase activity in prostate glands are different according to the lobe or part of the gland used. Furthermore, they demonstrated that the ratio of the zinc content to the enzyme activity is not constant in the glands as it is in the blood. Gunn *et al.* (11) and Kar *et al.* (12) observed the hormonal influence on the uptake of radioactive zinc by the dorsolateral prostates of rats. Our results, which were observed from the view point of carbonic anhydrase activity in the ventral prostates do not coincide with their results, but the functional differences in the lobes of the prostate glands should be considered.

According to Stafford *et al.* (13) the concentrations of acid and alkaline phosphatase in the seminal vesicles and prostates are affected by androgens. The behavior of those enzymes in castration or testosterone treatment is not similar to that of carbonic anhydrase. These chemical approaches should contribute to the knowledge of the functional state of the accessory organs of reproduction.

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EFFECT OF BOVINE PITUITARY GROWTH HORMONE ON HEPATIC CHOLESTEROGENESIS OF HYPOPHYSECTOMIZED RATS[1]

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ABSTRACT

Two groups of hypophysectomized rats were studied. In one group fed a stock diet, hepatic cholesterogenesis, as judged by the conversion of acetate carbon to cholesterol by slices of their livers, was subnormal. In the second group of hypophysectomized rats, hepatic cholesterogenesis was made to proceed at normal rates by the feeding of a diet containing 60 per cent glucose.

Beef pituitary growth hormone restored to normal the extent of acetate incorporation into cholesterol by the livers of hypophysectomized rats fed the stock diet. In those fed the 60 per cent glucose diet, the injection of the growth hormone increased above normal the liver's capacity for converting acetate carbon to cholesterol. This response to growth hormone is rapid—an increase in hepatic cholesterogenesis was observed as early as 6 hours after a single intravenous injection of the hormone.

Single intravenous injections of 100 μg. of the growth hormone as well as repeated daily intraperitoneal administrations of that amount did not increase hepatic cholesterogenesis in normal rats.

IN 1952 Tomkins *et al.* (1) reported that the conversion of acetate carbon to cholesterol was depressed in the liver of the hypophysectomized rat that had been fed a stock diet adequate in all respects and containing ground whole wheat as the main carbohydrate source. Subsequently it was observed that hepatic cholesterogenesis was normal in such a rat provided it was fed a diet containing 60% glucose (2). It was therefore concluded that maintenance of normal hepatic cholesterogenesis does not require the concurrence of anterior pituitary hormones.

The present report deals with the effects of growth hormone on hepatic cholesterogenesis. It is shown here that regardless of which diet was fed—the one with ground whole wheat as the main carbohydrate source or that containing 60% glucose—growth hormone administrations increased the capacity of the liver of the hypophysectomized rat to convert the C^{14} of acetate-1-C^{14} to cholesterol. Under the particular conditions of our study, the liver of the normal rat failed to respond to growth hormone administrations.

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EXPERIMENTAL

Rats of the Long-Evans strain, weighing from 120 to 140 gm., were hypophysectomized by the parapharyngeal route. They were used 2 to 4 weeks after hypophysectomy. At the end of the experiments the heads of the operated animals were examined under a dissecting microscope to determine completeness of pituitary removal. The findings reported here were obtained with operated animals in which no remnant of the pituitary gland or gross damage to the hypothalamic area was observed. Normal rats of the same weight range served as controls.

All rats were raised and maintained on a stock diet in which ground whole wheat constituted the main carbohydrate source. In some cases the rats were transferred, for several days before they were killed, to a synthetic diet containing 60 per cent glucose. The composition of the two diets is given in Table 1.

Three highly purified beef pituitary growth hormone preparations were used: 1) somatotrophin, No. 491276, obtained from Armour Laboratories; 2) preparation No. L-2582-KB kindly furnished by Dr. C. H. Li (4) of the Hormone Research Laboratory of this University; and 3) a preparation kindly supplied by Dr. S. Ellis of the Institute of Experimental Biology of this University. The two latter preparations have been shown to be homogeneous by electrophoretic and other physicochemical methods, and free of adrenocorticotrophic activity by the Sayers test. The Armour preparation was dissolved in distilled water; the other two were dissolved in saline adjusted to pH 9–10 with NaOH. Fresh solutions of the hormones (100 μg. per 0.1 ml.) were prepared every second day. Control animals were injected with the solvent alone.

The preparation of liver slices, their incubation with acetate-1-C^{14}, and the methods for determination of C^{14} in respired CO_2 and cholesterol digitonides have been described (2). Total liver cholesterol was determined by the method of Sperry and Webb (5).

RESULTS

1. *Experiments with rats fed the stock diet and injected intraperitoneally with 50–250 μg. of growth hormone daily for 6 days*

It was again evident, as reported earlier (1, 2), that the livers of hypophysectomized rats (Table 2) fed the diet in which ground whole wheat constituted the main carbohydrate source had a reduced capacity for converting acetate carbon to cholesterol. The treatment of hypophysectomized rats with 50, 100, or 250 μg. of growth hormone daily for 6 days restored to normal the extent to which acetate carbon was incorporated into cholesterol by slices prepared from the livers of these rats.

TABLE 1. COMPOSITION OF DIETS

Constituent	Labration*	Synthetic diet†
	Per cent	Per cent
Carbohydrate	46.5	60
Protein	24.7	22
Fat	5.2	0
Fiber	4.0	9.8

* A nutritionally complete diet obtained from Diablo Animal Laboratories, Berkeley, California.

† In addition to the constituents listed above, this diet contained 2.0 per cent of liver VioBin, 0.2 per cent of a vitamin B mixture (2) and 6.0 per cent of Hawk-Oser salt mixture (3). Vitamins A and D were given every third day when the synthetic diet was fed for more than three days.

TABLE 2. CHOLESTEROL SYNTHESIS IN LIVER SLICES PREPARED FROM HYPOPHYSECTOMIZED RATS FED THE STOCK DIET AND INJECTED INTRAPERITONEALLY WITH GROWTH HORMONE DAILY FOR 6 DAYS

Duplicate 500-mg. portions of each liver were incubated with 2 μmoles of carboxyl labeled acetate for 3 hours at 37.5° in a Krebs-Henseleit bicarbonate buffer (pH 7.3). The gas phase was 95 per cent O_2 and 5 per cent CO_2. Each liver portion was analyzed separately

Rats		Growth hormone		Average wt. gain in 6 days	Liver total cholesterol		Per cent of added acetate-C-14 recovered as cholesterol	
State	Number	Preparation	Amount injected daily		Range	Average	Range	Average
			μgm.	gm.	per cent wet weight	per cent wet weight		
Normal	5	None			0.14–0.22	0.18	1.4–3.0	2.3
HypX*	5	None		0	0.16–0.35	0.25	0.04–0.40	0.15
HypX	2	A†	50	9	0.17–0.19	0.18	1.0–2.1	1.6
HypX	1	A	250	11		0.21		1.4
HypX	4	L§	50	11	0.13–0.24	0.20	1.6–3.6	2.2
HypX	2	L	100	14	0.19, 0.24	0.22	2.2, 4.7	3.5
HypX	2	E‡	50	12	0.21, 0.23	0.22	1.8, 2.6	2.2
HypX	2	E	250	15	0.23, 0.24	0.24	2.1, 2.8	2.5

* Hypophysectomized.
† Obtained from Armour Laboratories.
§ Obtained from Dr. C. H. Li of the Hormone Laboratory, University of California, Berkeley.
‡ Obtained from Dr. S. Ellis of the Institute of Experimental Biology, University of California, Berkeley.

2. *Experiments with rats fed the stock diet and sacrificed 6 hours after a single intravenous injection of 100 μg. of growth hormone*

Table 3 shows that the stimulating effect of growth hormone on hepatic cholesterogenesis in the hypophysectomized rat appears as early as 6 hours after a single intravenous injection. The same amount of hormone, however, failed to induce a rise in the normal rat.

3. *Experiments with hypophysectomized rats fed the 60 per cent glucose diet and sacrificed 6 hours after a single intravenous injection of growth hormone*

The preceding findings with normal rats raised the interesting question of whether the stimulating effect of growth hormone was restricted to livers with lowered cholesterogenic capacities. For this reason we also studied the action of growth hormone on hepatic cholesterogenesis in hypophysectomized rats that had been fed the 60 per cent glucose diet for 3 days. The livers of hypophysectomized rats so fed, it should be noted, have a normal capacity for converting acetate carbon to cholesterol (2). The results recorded in Table 4 show that growth hormone is also effective in the hypophysectomized rat even when hepatic cholesterogenesis is not impaired.

The C^{14}-cholesterol recoveries in the experiments with livers of the normal controls of this experiment (Table 4) were much lower than those observed with the normal control rats in experiments 1 (Table 2) and 2 (Table 3). This probably reflects the difference in the fat content of the two

TABLE 3. CHOLESTEROL SYNTHESIS IN LIVER SLICES PREPARED FROM NORMAL AND HYPOPHYSECTOMIZED RATS FED THE STOCK DIET AND SACRIFICED 6 HOURS AFTER A SINGLE INTRAVENOUS INJECTION OF GROWTH HORMONE

For experimental details see Table 2. The growth hormone preparation was obtained from Dr. C. H. Li.

Rats		Growth hormone injected	Liver total cholesterol		Per cent of added acetate-C^{14} recovered as cholesterol	
State	Number		Range	Average	Range	Average
		μgm.	per cent wet weight	per cent wet weight		
Normal	4	None	0.17–0.23	0.20	2.6–3.1	2.9
Normal	4	100	0.14–0.23	0.19	1.4–2.0	1.7
HypX	3	None	0.24–0.33	0.27	0.05–0.50	0.25
HypX	5	100	0.14–0.23	0.19	1.4–2.1	1.6

diets (Table 1). We have shown elsewhere (6) that the addition of fat to the diet increases the liver's capacity for converting acetate carbon to cholesterol.

4. *Experiments with normal rats fed the 60 per cent glucose diet and sacrificed from 6 hours to 11 days after the first daily injection of 100 μg. of growth hormone*

The design and results of these experiments are shown in Table 5. Even under this dietary condition a single intravenous injection or several daily intraperitoneal injections of the hormone failed to elicit an increase in hepatic cholesterogenesis of the normal rat.

5. *Time and concentration studies with rats fed the 60 per cent glucose diet for 3 days and sacrificed 6 hours after a single intravenous injection of 100 μg. of growth hormone*

In the preceding experiments the liver slices were incubated with 2 μmoles of acetate for exactly 3 hours. In other experiments, the results of

TABLE 4. CHOLESTEROGENESIS IN SLICES PREPARED FROM THE LIVERS OF HYPOPHYSECTOMIZED RATS FED THE 60 PER CENT GLUCOSE DIET AND SACRIFICED 6 HOURS AFTER A SINGLE INTRAVENOUS INJECTION OF GROWTH HORMONE

For experimental details see Table 2. The growth hormone preparation was obtained from Dr. C. H. Li.

Rats		Growth hormone injected	Per cent of acetate-C^{14} recovered as:			
			CO_2		Cholesterol	
State	Number		Range	Average	Range	Average
		μgm.				
Normal	6	None	31–52	40	0.5–1.3	1.0
HypX	7	None	31–54	38	0.7–1.5	1.0
HypX	12	100	37–59	49	2.0–6.8	4.0

TABLE 5. CHOLESTEROGENESIS IN LIVER SLICES OF NORMAL RATS FED THE 60 PER CENT GLUCOSE DIET AND INJECTED WITH GROWTH HORMONE

For experimental details see Table 2. The hormone preparation was obtained from Dr. C. H. Li. The rats were fed the 60 per cent glucose diet for 3 days before the start and throughout the duration of the experiment.

Number of rats	Growth hormone injected		Time sacrificed after 1st injection	Per cent of acetate-C^{14} recovered as cholesterol	
	Amount	Route		Range	Average
	μgm.				
5	None			0.7–1.9	1.4
8	100	Intravenous	6 hours	0.4–1.9	1.3
3	100	Intravenous	24 hours	0.5–1.3	1.0
3	100*	Intraperitoneal	3 days	1.6–1.8	1.7
3	100*	Intraperitoneal	7 days	1.3–2.1	1.6
3	100*	Intrapertioneal	11 days	0.7–1.7	1.2

* This amount of hormone was injected daily.

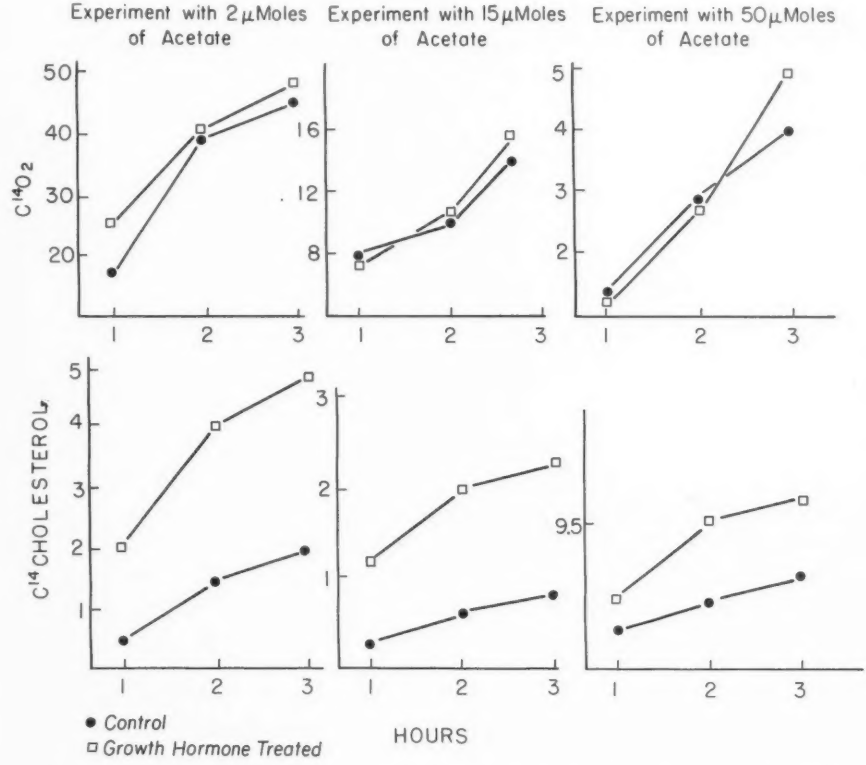

FIG. 1. Time and concentration study with slices prepared from livers of hypophysectomized rats. For explanation see text. The ordinate values are percentages of the C^{14} added to the incubation medium.

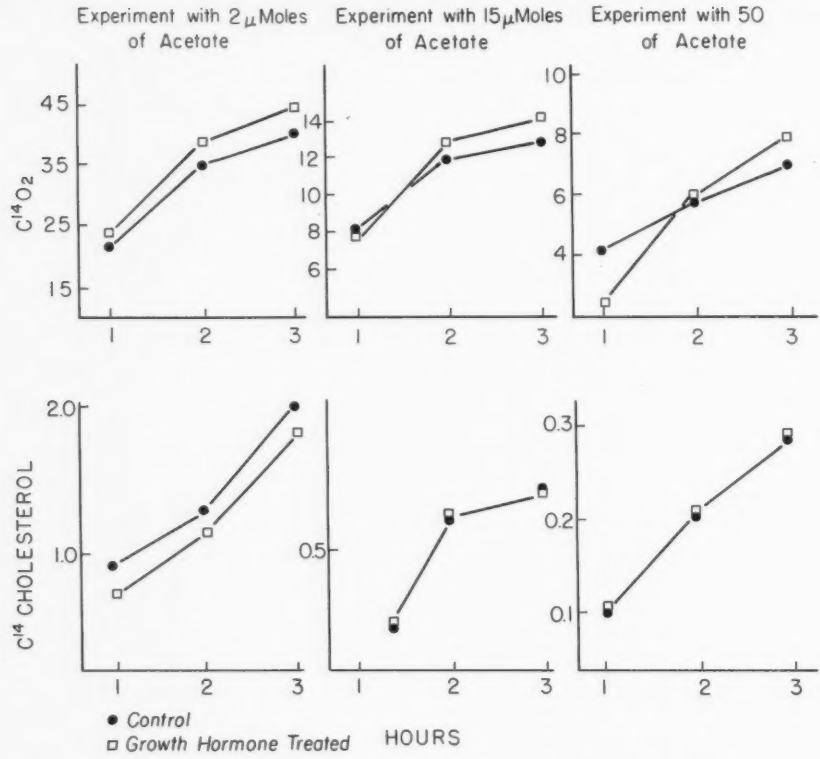

FIG. 2. Time and concentration study with slices prepared from livers of normal rats. For explanation see text. The ordinate values are percentages of the C^{14} added to the incubation medium.

which are shown in Figures 1 and 2, separate pools of slices were prepared from the livers of a) 3 hypophysectomized rats that had been injected with growth hormone, b) 3 normal rats treated exactly the same way, c) 3 hypophysectomized rats injected with saline, and d) 3 normal rats injected with saline. Separate 500-mg. portions of each pool were incubated for 1, 2, and 3 hours with 2, 15, and 50 μmoles of labeled acetate, respectively. The stimulation of hepatic cholesterogenesis of the hypophysectomized rat by growth hormone was noted at the early intervals, as well as at 3 hours, with all 3 concentrations of acetate in the medium. No such effect of the hormone was found in the experiments with slices prepared from the livers of normal rats. In no case—with either hypophysectomized or normal rats—did growth hormone measurably affect the $C^{14}O_2$ recoveries.

DISCUSSION

In the present investigation we used preparations obtained from three sources in order to study the effect of bovine growth hormone on hepatic cholesterogenesis in hypophysectomized rats. Since as little as 25 μg. of two of the growth hormone preparations induced normal growth rates in hypophysectomized rats, the amount of hormone injected daily was not allowed to exceed 250 μg. As little as 50 μg. of the hormone restored hepatic cholesterogenesis to normal in hypophysectomized rats fed the ground whole wheat diet. In hypophysectomized rats in which hepatic cholesterogenesis was first restored to normal by dietary means, the injection of 100 μg. of growth hormone raised hepatic cholesterogenesis to levels above those observed in the normal. The response of the hypophysectomized rat to the hormone is rapid, and an effect was observed by the time 6 hours had elapsed after a single intravenous injection of 100 μg.

Under the conditions of our experiments, injections of 100 μg. of growth hormone even for several days failed to stimulate hepatic cholesterogenesis in the normal rat. Apparently, in the presence of an intact pituitary gland, hepatic cholesterogenesis is insensitive to exogeneous growth hormone. Other investigators (7, 8, 9), however, have shown that very large doses of growth hormone—from 1 to 15 mg.—can increase the capacity of normal rat liver to convert acetate carbon to cholesterol.

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EFFECTS OF o,p′ DDD[1] ON HISTOLOGY AND 17-HYDROXYCORTICOSTEROID OUTPUT OF THE DOG ADRENAL CORTEX

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ABSTRACT

In dogs receiving oral treatment with 2,2-bis (2-chlorophenyl-4-chlorophenyl)-1,1-dichloroethane (o,p′ DDD) for periods of 2, 4, and 6 days, the secretion rate of 17-hydroxycorticosteroids was markedly decreased. Histological observations on the adrenal cortex showed that focal degenerative lesions developed first in the zona reticularis, then in the zona fasciculata. No changes were seen in the zona glomerulosa even after 6 days treatment. Morphological changes in the zona reticularis and zona fasciculata were slight after 2 days treatment although there was already a marked reduction in the digitonin-precipitable compounds of these zones. After 6 days treatment, 3β-hydroxy sterols were almost absent from the zona fasciculata and zona reticularis. Distribution of sudanophilic lipids of these zones remained unaltered during this period.

RECENT investigations have indicated that o,p′ DDD, an isomer found in technical DDD, produces certain alterations of adrenocortical function previously associated with the technical compound (1, 2).

In the present experiments, we have been concerned with functional and histological alterations of the dog adrenal cortex following short-term oral treatment with o,p′ DDD. The following observations provide direct evidence for a decreased rate of 17-hydroxycorticosteroid secretion by the adrenals together with correlated descriptions of histological features and histochemical distribution patterns of digitonin-reacting sterols in the adrenal cortex.

METHODS

Twenty adult male mongrel dogs maintained on a standard commercial ration were used in these studies. Body weight ranged from 10 to 15 kg. Animals were housed individually in an area maintained at temperatures of 22 to 26° C. Sesame oil solutions of o,p′ DDD in gelatin capsules were administered daily by mouth for periods of 2 to 6 days. At 20 to 24 hours following the last dose of o,p′ DDD, Nembutal was given intravenously (27.0 mg. Nembutal per kg. body weight). The right lumbo-adrenal vein was cannulated according to the procedure previously described by Hume and Nelson

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[1] 2,2-bis (2-chlorophenyl-4-chlorophenyl)-1,1-dichloroethane.

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(3). Normal body temperature was maintained by electric heating pads. Adrenal venous blood samples were collected in triplicate for 10 minute periods with intervals between each sample. Samples were immediately refrigerated. The method of Silber and Porter, as modified by Peterson *et al.* (4), was used for determination of 17-hydroxycorticosteroids in whole blood.

After collection of the final blood sample, the animal was sacrificed and the left adrenal gland removed immediately for histological and histochemical study. Alternate pieces of each gland were fixed in Bouin's solution and Baker's calcium-formol solution (5). Paraffin sections were stained by the following techniques: Mayer's hemalum and eosin; Crossmon's modification of Mallory's stain (6); allochrome (7); periodic acid-Schiff (8) controlled with sections previously treated for 1 hour with 0.5% solution of Taka-diastase (Parke-Davis) in phosphate buffer at pH 6. Frozen sections were treated with a solution of Sudan black B in 70% alcohol (9) to demonstrate lipids and with an alcoholic solution of digitonin (10) which reacts specifically with 3β-hydroxy sterols, such as cholesterol, to form insoluble digitonides. The latter were observed with polarized light.

RESULTS AND DISCUSSION

Table 1 summarizes results of short-term experiments on dogs receiving o,p′ DDD orally. The histology of the adrenal cortex and the rates of 17-hydroxycorticosteroid secretion of 13 treated dogs are compared with similar parameters in 7 untreated controls.

A. Adrenal Cortical Histology

Lesions were already present in the adrenal cortex of animals treated with o,p′ DDD for 2 days (Fig. 2). The cells were less acidophilic than those of the untreated dog (Fig. 1). Large vacuoles, indicating the presence of lipid were apparent in the cytoplasm of many cells. The nuclei were smaller and more hyperchromatic. There were occasional foci of cellular atrophy, pyknosis and nuclear fragmentation denoting marked degenerative changes. Lymphocytic infiltration was also evident in these areas. These foci of cellular atrophy were observed in the zona reticularis although some were seen in the zona fasciculata. No changes were apparent in the zona glomerulosa.

After 6 days of treatment, the degenerative phenomena were more intense and diffuse (Fig. 3). The zona reticularis was markedly affected. The zona fasciculata was abnormal in that the trabecular arrangement was not as well defined as in the controls and one could see leucocytic infiltration even in the septa. The cortex had decreased in width. There was little change in the zona glomerulosa except that a few cells showed vacuolization. The absence of lesions in the zona glomerulosa may be due to the short duration of these experiments.

Collagen staining showed that there was an apparent fibrosis in the juxta-medullary region. This alteration was produced by the disappearance of the glandular tissue and condensation of the pre-existing stroma.

Staining with Sudan black B demonstrated that the cellular lipids may

TABLE 1. EFFECT OF o,p′ DDD ON ADRENAL 17-HYDROXYCOSTICOSTEROIDS AND HISTOLOGY

Dog No.	Daily Dose o,p′ DDD (mg. per kg.)	Adrenal Vein 17-OH Corticosteroids μg. per min.	ADRENAL CORTEX	
			HISTOLOGY	DIGITONIN REACTION
			Untreated	
1	0	6.4, 6.3, 7.5	Normal	Normal
2	0	10.5, 10.7, 9.0	Normal	Normal
3	0	8.8, 7.5	Normal	Normal
4	0	7.0, 8.5, 7.2	Normal	Normal
5	0	4.5, 4.7, 5.8	Normal	Normal
6	0	5.5, 5.5	Normal	Normal
7	0	9.2, 9.6	Normal	Normal
			2 Days Treatment	
8	100.0	0.4, 0.6, 0.4	Glomerulosa unchanged. Foci of necrosis and leukocytic infiltration abundant in reticularis, less in fasciculata.	Partial depletion of digitonin precipitate in fasciculata. No precipitate in reticularis.
9	100.0	0.6, 0.8	Similar to preceding. Lesions less intense.	Slightly augmented in glomerulosa. Focal depletion in reticularis and fasciculata.
10	100.0	2.0, 1.9, 1.9	Small isolated foci in reticularis. No change in glomerulosa and fasciculata.	Digitonin precipitate slightly augmented in glomerulosa; relatively little in fasciculata, none in reticularis.
			4 Days Treatment	
11	50.0	6.4, 6.4, 7.5	Normal	Distribution normal.
12	50.0	0.5, 0.3, 0.4	Small foci of leucocytosis and necrosis in reticularis. Glomerulosa and fasciculata not unusual. Lesions less marked then in 2 preceding cases.	Slight depletion in reticularis and fasciculata.
			6 Days Treatment	
13	5.0	6.9, 6.5, 6.3	Normal	Normal distribution.
14	5.0	4.4, 5.0, 5.0	Normal	Augmented in glomerulosa. Fasciculata and reticularis normal.
15	25.0	0.5, 0.5, 0.5	Intense lesions of reticularis and fasciculata, reticularis hemorrhagic.	Normal in glomerulosa. None in reticularis and fasciculata.
16	25.0	0.9, 0.8, 0.9	Similar to preceding.	Similar to preceding.
17	40.0	0.8, 0.7, 0.6	Foci of leucocytic infiltration in reticularis and fasciculata, vacuolization of cells in fasciculata. Less intense lesions than in 2 preceding.	Almost completely absent from reticularis and fasciculata. Normal glomerulosa.
18	40.0	0.7, 0.7, 0.4	Similar to preceding. Lesions less pronounced.	Similar to preceding.
19	50.0	0.5, 0.5, 0.3	Extensive marked lesions with some vacuolization of cells in glomerulosa. Intense lymphocytic infiltration.	Reduced in amount and completely absent in scattered foci in reticularis and fasciculata. Glomerulosa normal.
20	50.0	0.3, 0.3	Degenerative lesions in fasciculata and reticularis. Hemorrhage in reticularis.	Glomerulosa normal. Absent in fasciculata and reticularis.

first appear to be normal or slightly augmented. Treatment for 2 to 6 days (Fig. 8) did not produce noteworthy changes in distribution of sudanophilic lipids.

The digitonin reaction indicated that the distribution of 3β-hydroxy sterols in the adrenal cortex of the untreated dog (Fig. 4) was similar to that of the sudanophilic lipids (Fig. 7). Except for a narrow band corresponding to the intermediate zone, the reaction was positive throughout the cortex. Following 2 days of treatment with o,p′ DDD, depletion of the digitonin precipitate was seen in the zona reticularis and zona fasciculata although the zona glomerulosa remained unchanged (Fig. 5). The alteration in the digitonin reaction was evident even before morphological lesions became prominent. After 6 days of treatment, the 3β-hydroxy

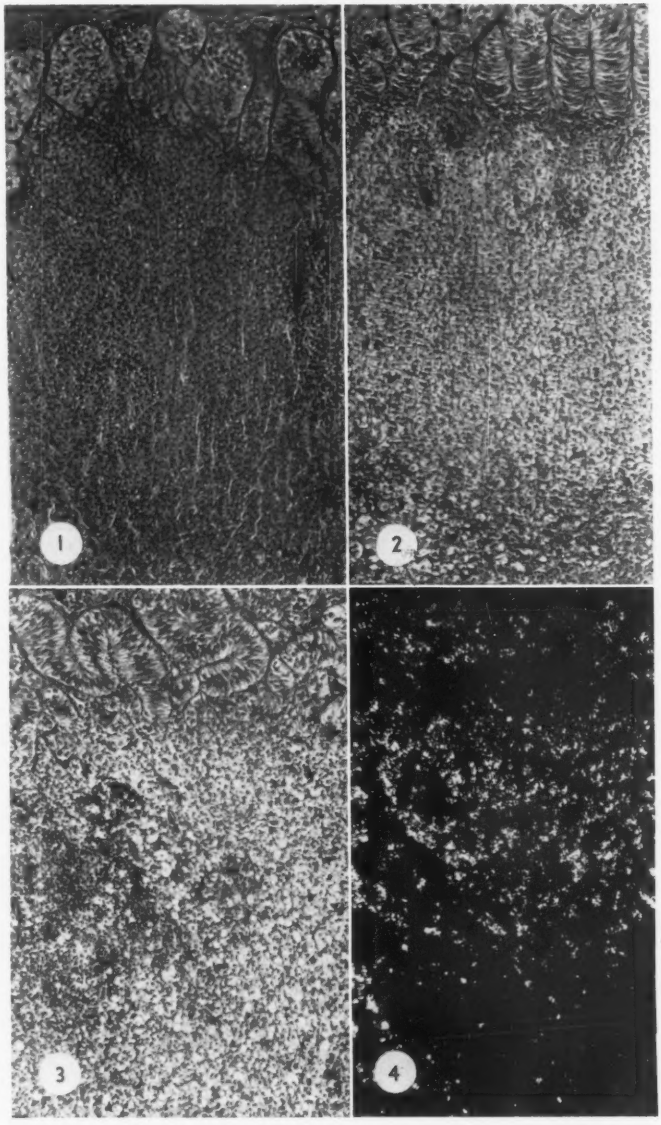

FIG. 1. Adrenal Cortex of normal male dog No. 2 (hematoxylin and eosin).

FIG. 2. Adrenal cortex of male dog No. 8 treated for 2 days with o,p′ DDD (hematoxylin and eosin).

FIG. 3. Adrenal Cortex of male dog No. 17 treated for 6 days with o,p′ DDD (hematoxylin and eosin).

FIG. 4. Adrenal cortex of normal male dog No. 2, digitonin reaction for 3β-hydroxy sterols.

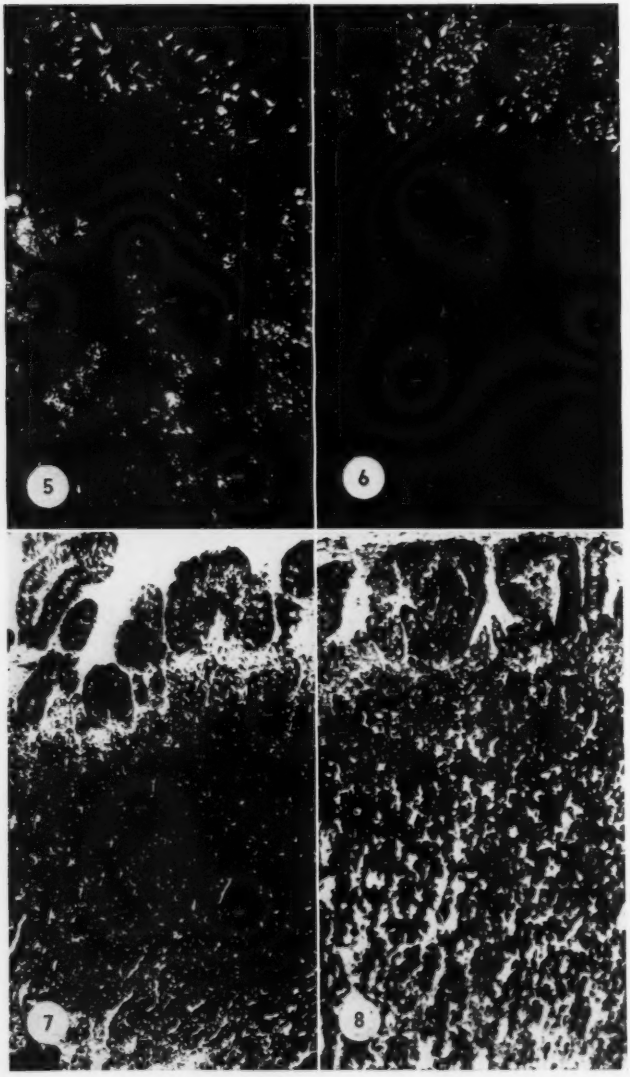

FIG. 5. Adrenal cortex of male dog No. 8, treated for 2 days with o,p′ DDD, digitonin reaction for 3β-hydroxy sterols.

FIG. 6. Adrenal cortex of male dog No. 17, treated for 6 days with o,p′ DDD, digitonin reaction for 3β-hydroxy sterols.

FIG. 7. Adrenal cortex of normal male dog No. 2, Sudan black B for sudanophilic lipids.

FIG. 8. Adrenal cortex of male dog No. 17, treated for 6 days with o,p′ DDD, Sudan black B for sudanophilic lipids.

sterols were almost entirely absent from the zona reticularis and zona fasciculata with little change in the zona glomerulosa (Fig. 6).

As noted previously, the reaction with Sudan black B showed no marked alteration of lipid distribution although there is normally a close correspondence between the depletion of digitonin precipitable steroids and the density of sudanophilic lipids (11).

Anatomical lesions of the adrenal cortex were not observed in 2 dogs (No. 13 and 14) receiving daily doses of only 5 mg. per kg. of o,p′ DDD for 6 days and a third dog (No. 11) treated for 4 days at 50 mg. per kg. per day.

B. Adrenal Vein 17-Hydroxycorticosteroid Secretion

A marked decrease in the minute ouput of 17-hydroxycorticosteroids was seen after 2 days of treatment with o,p′ DDD (100 mg. per kg. per day). When the duration of drug administration was increased to 4 days and the dose decreased to 50 mg. per kg. per day, secretion of 17-hydroxycorticosteroids was diminished in one of 2 treated animals.

After 6 days of treatment with o,p′ DDD at doses ranging from 25 mg. per kg. per day to 50 mg. per kg. per day the secretion rate of 17-hydroxycorticosteroids was markedly reduced. In the 2 dogs receiving only 5 mg. per kg. per day, values were within the normal range.

Intravenous administration of 1 USP unit of ACTH, which normally elicits a maximal rise in 17-hydroxycorticosteroid output in the hypophysectomized dog, failed to alter the low secretion rate in the dogs treated with o,p′ DDD (100 mg. per kg. per day) for 2 days.

The failure of ACTH to increase output of 17-hydroxycorticosteroids in spite of minimal cortical lesions indicates a direct effect of o,p′ DDD on adrenocortical function rather than an effect of the drug on the anterior pituitary.

Previous studies have demonstrated that the simultaneous administration of ACTH and technical DDD does not prevent the adrenocorticolytic action of the latter (12). Moreover, following chronic treatment with technical DDD, ACTH had no effect on the low rate of 17-hydroxycorticosteroid secretion either before or after hypophysectomy (13).

In the 10 treated dogs in which adrenocortical function was altered in response to treatment with o,p′ DDD, the severity of the cortical lesions observed histologically increased with the duration of drug administration. However, when cortical histology is compared with the corresponding rate of 17-hydroxycorticosteroid secretion it is apparent that the degree of morphologic alteration cannot be used to assess cortical function, for even in the presence of minimal histological lesions, the output of 17-hydroxycorticosteroids was reduced to levels previously observed in this laboratory in the acutely hypophysectomized dog (13).

Acknowledgments

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EFFECT OF CORTISONE, 9-ALPHAFLUOROCORTISOL, AND GROWTH HORMONE ON THE ACTION OF TOLBUTAMIDE[1]

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ABSTRACT

Rats were treated for 10 days with cortisone acetate, or with 9-alphafluorocortisol acetate. The response in these animals, to a single oral dose of tolbutamide sodium was compared with that in untreated controls. In the animals pretreated with the corticosteroids, the tolbutamide-induced hypoglycemia was greatly diminished in rats fed a stock diet (Purina Laboratory chow); in similarly treated animals fed a high carbohydrate diet (75%), administration of tolbutamide was followed by hyperglycemia in many animals. The response of the individual animals was not correlated to the presence of glycosuria, or of fasting hyperglycemia. In rats pretreated with cortisone, the response to insulin (4 U.) did not differ from that of controls. Pretreatment with growth hormone slightly, but significantly intensified the tolbutamide-induced hypoglycemia. Mechanisms possibly involved are discussed, but at present no satisfactory explanation can be offered.

THE action of sulfonylureas in carbohydrate metabolism has been studied extensively in recent years. However, few investigations have dealt with possible modifications of the effects of sulfonylureas by the endocrine system. The blood sugar lowering effect of the sulfonylureas can be obtained in the absence of the adrenals or of the pituitary gland (1). In the adrenalectomized cat (2), and rat (3), the hypoglycemic response to tolbutamide is greater than in intact animals. The suppression of some parameters of thyroid function induced by these compounds (4, 5) is probably an ancillary effect, which neither mediates nor modifies the action upon carbohydrate metabolism.

In this paper we report studies on the effect of tolbutamide (Orinase) in rats treated with growth hormone and with cortisone or 9-alphafluorocortisol.

MATERIAL AND METHODS

In all experiments male rats (Sprague Dawley-descendants obtained from Barkbridge Farms, N. J.) weighing between 200 and 250 gm. were used. They were fed either stock diet (Purina Laboratory Chow) or a high carbohydrate diet.[2] The animals

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[2] The high carbohydrate diet had the following composition: Casein 17%, glucose 75%, corn oil 4%, salt mixture (USP ×111) 4%. To each 1000 gm. of this diet the

were fasted for 26 or 48 hours prior to the administration of tolbutamide. A blood sample was drawn by cardiac puncture (referred to subsequently as pretolbutamide sample, or as sample at time zero); immediately afterwards 100 mg. of tolbutamide sodium (Orinase-sodium) in aqueous solution was administered by stomach tube. The animal was then anesthetized by intraperitoneal injection of nembutal (30 mg./kg.) and blood samples were obtained by heart puncture at 1, 2, 3, and 4 hours after the administration of tolbutamide. Clotting was prevented in all blood samples by adding a small amount of heparin powder. Blood sugar determination was carried out by the micromethod of Somogyi-Nelson (6). Blood sugar changes are reported in per cent of the value obtained before administration of tolbutamide (zero hour). Growth hormone, cortisone acetate or 9 alphafluorocortisol acetate were injected daily for 10 days prior to the administration of tolbutamide sodium. The daily dose of cortisone acetate was 5 mg., that of 9-alphafluorocortisol acetate 1.25 or 2.5 mg. and that of growth hormone 5 mg.

RESULTS

Influence of cortisone acetate, and of 9 alphafluorcortisol on response to tolbutamide

Following the oral ingestion 100 mg. of tolbutamide sodium, the blood sugar decreases; the blood sugar values are slightly lower in animals fed a high carbohydrate diet prior to administration of tolbutamide than in the rats fed a stock diet, but in the former they rise somewhat at 3 and 4 hours.

In animals fed the stock diet, and pretreated with cortisone acetate for 10 days (5 mg./day), a single dose of tolbutamide sodium is followed by hypoglycemia of short duration (one hour) with subsequent rise of blood sugar values to, or above the pretolbutamide level (Fig. 1). Statistically, in the cortisone-group, the hypoglycemic level at one hour is significantly different (logarithmic analysis of per cent changes) from the level at time 0 ($P < 0.001$), whereas the mean blood sugar levels at 2, 3 and 4 hours are not significantly different from the value at time 0. In the control groups the differences at all times are significantly lower than at time 0. Comparison of the control- and cortisone groups shows that at one hour the hypoglycemic level in the two groups does not differ significantly, whereas the differences at 2, 3 and 4 hours are significant ($P < 0.05$, < 0.001, < 0.001 respectively).

Rats fed the high carbohydrate diet were pretreated with cortisone acetate (5 mg./day), or with 9 alphafluorocortisol (1.25 or 2.5 mg.) for 10 days. Results are given in Figure 2. In contradistinction to the control groups, the mean blood sugar values in the groups pretreated with the adrenocortical steroids show no significant decrease and a tendency to rise

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following vitamin supplements were added: riboflavin 2.5 mg., thiamine HCl 1.2 mg., Ca pantothenate 10.0 mg., pyridoxine 1.0 mg., choline chloride 1000 mg., nicotinic acid 6.3 mg., inositol 25.0 mg., biotin 2.5 mg., Vit. K. (Menadion) 2.5 mg., Vit. B_{12} 0.1 mg., Vit. E (alphatocopherol) 50 mg., codliver oil 5.0 ml.

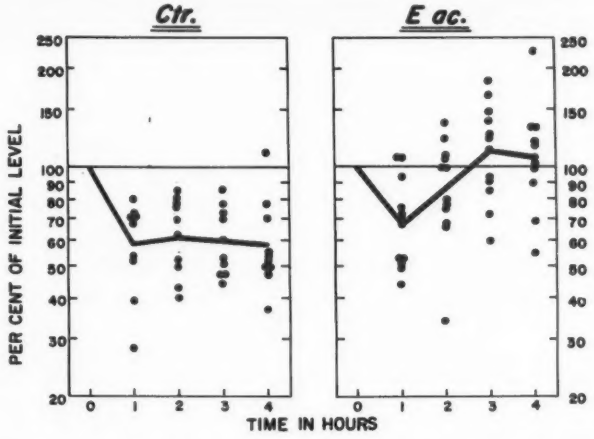

FIG. 1. Effect of pretreatment with cortisone acetate on hypoglycemic action of tolbutamide. Diet: Purina laboratory chow. Blood sugar values in per cent of value before tolbutamide (plotted logarithmically). Tolbutamide sodium given after the time zero blood sample was drawn. Left curve: controls, right curve: pretreatment with cortisone acetate. Statistical evaluation: see text.

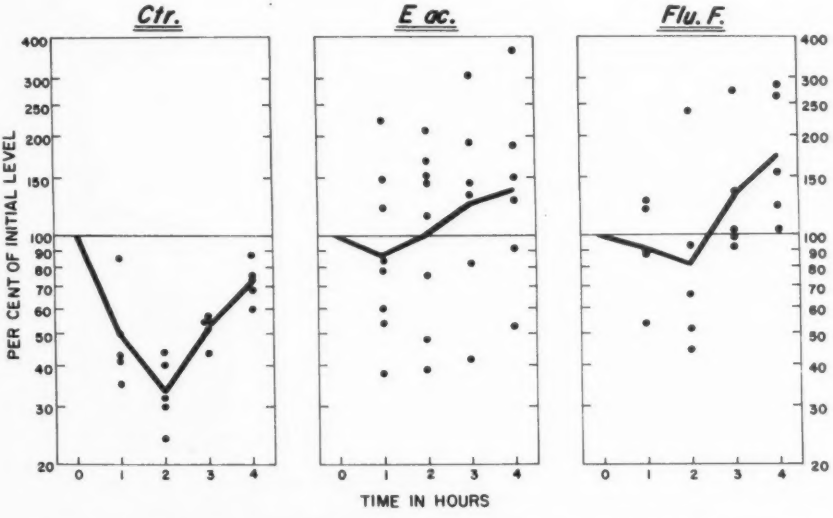

FIG. 2. Effect of pretreatment with cortisone acetate, and with 9-alphafluorocortisol acetate, on hypoglycemic action of tolbutamide. Diet: high carbohydrate. Blood sugar values in per cent of value before tolbutamide (plotted logarithmically). Tolbutamide sodium given after the time zero blood sample was drawn. Left curve (Ctr.): controls; middle curve (E.ac.): pretreated with cortisone acetate; right curve (FluF): pretreated with 9-alphafluorocortisol acetate. Statistical evaluation: see text.

to hyperglycemic values; this rise is very marked in some animals, but the mean reaches statistical significance (P 0.05) only in the 9 alpha-fluorocortisol treated animals at 4 hours. Statistically, there is no difference at any time between the animals pretreated with cortisone acetate and those with 9 alphafluorocortisol. On the other hand, these two groups combined, differ significantly from the control group at every time ($P < 0.05$, < 0.01, < 0.01, < 0.05 respectively at 1, 2, 3, and 4 hours).

The response of individual animals to tolbutamide is quite uniform in the control groups, whereas the animals pretreated with corticosteroids show a considerable variability of response. Some animals having received cortisone acetate, and four out of five having received 9-alphafluorocortisol acetate, had fasting hyperglycemia, and mild glycosuria, but the individual response to tolbutamide was not correlated to the presence of these indicators of steroid-induced diabetes.

Influence of cortisone acetate on response to insulin

In contradistinction to the impairment, or abolition of the hypoglycemic response to tolbutamide observed after pretreatment with 9-alphafluorocortisol and with cortisone acetate, pretreatment with the latter failed to influence significantly the response to a single subcutaneous dose of 4 units of insulin (Table 1).

Influence of growth hormone on response to tolbutamide

In rats pretreated with daily subcutaneous injections of 5 mg. of beef growth hormone for 10 days, the hypoglycemic response to tolbutamide was somewhat enhanced; during the first three hours, the means of the blood sugar levels were lower than in the non-pretreated controls receiving tolbutamide (Table 2). The difference was statistically significant at 2 and 3 hours after administration of tolbutamide (t'' test on arithmetic means).

DISCUSSION

The experiments reported in this paper show that pretreatment of rats with cortisone acetate or with 9-alphafluorocortisol acetate greatly dimin-

TABLE 1. EFFECT OF PRETREATMENT WITH CORTISONE ACETATE ON HYPOGLYCEMIC ACTION OF INSULIN (4 U). BLOODSUGAR VALUES IN PER CENT OF VALUE BEFORE INSULIN, WITH STANDARD ERROR OF MEAN. DIET: HIGH CARBOHYDRATE

	Blood sugar			
	Hours after insulin			
	1	2	3	4
Controls (6)*	100	31 ± 5.75	27 ± 4.23	27 ± 3.21	20 ± 3.05
Cortisone ac. 5 mg./day × 10 (6)*	100	49 ± 7.72	33 ± 6.77	29 ± 5.88	19 ± 6.19

* Number of animals.

TABLE 2. EFFECT OF PRETREATMENT WITH GROWTH HORMONE ON HYPOGLYCEMIC ACTION OF TOLBUTAMIDE SODIUM. BLOODSUGAR VALUES IN PER CENT OF VALUE BEFORE TOLBUTAMIDE ADMINISTRATION, WITH STANDARD ERROR OF MEAN. DIET: PURINA LABORATORY CHOW

	Blood sugar				
	Hours after tolbutamide				
	Before	1	2	3	4
Controls (10)*	100	60 ± 5.26	64 ± 5.2	61 ± 4.7	62 ± 6.6
Growth hormone 15 mg./day × 10 (13)*	100	47 ± 6.0	49 ± 4.6	45 ± 4.6	66 ± 4.9
P†		<0.2	<0.05	<0.02	

* Number of animals.
† Fisher's "Student" method.

ishes or abolishes the hypoglycemic action of a single dose of tolbutamide. In many animals tolbutamide administration is followed by hyperglycemia rather than by hypoglycemia. The response to a large dose of insulin is not modified by pretreatment with cortisone acetate. These observations add to the evidence that in many respects the actions of tolbutamide and of insulin differ; stimulation of insulin secretion by the drug, at one time considered as perhaps the essential mode of action of sulfonylureas in carbohydrate metabolism, cannot be the only, and perhaps not the most important effect of these agents.

There is little information as to whether, and to what extent, hormonal factors influence or modify the action of sulfonylureas. Houssay *et al.* have found these drugs to be active in the absence of the adrenal or the pituitary (1). Gordon *et al.* have reported that adrenalectomy, but not hypophysectomy greatly enhances the hypoglycemic action of tolbutamide in the cat (2); Williams has observed the same effect of adrenalectomy in the rat (3) and Volk *et al.* in the dog (7). Our experiments show the corollary, the abolition of the hypoglycemic effect of the drug by pretreatment with cortisone.

Species differences appear to exist in this respect in as much as Volk *et al.* (7) have found the cortisone treated rabbit to exhibit a good hypoglycemic response to tolbutamide. The dose of the latter drug was identical with that employed by us (500 mg./kg.), but the dose of cortisone was somewhat smaller (10 mg./kg. as against 20 to 25 mg./kg. in our experiments) Reports on the response of "steroid diabetes" in man, to sulfonylureas differ; Miller and Craig found tolbutamide effective in a case of Cushing's syndrome with diabetes (the patient receiving constant amounts of insulin) (8), and Moorhouse *et al.* obtained a good response to tolbutamide in a case of lupus erythematosus with diabetes, receiving daily 200 mg. of cortisol (9). In contradistinction Fajans *et al.* found tolbutamide ineffective in a case of Cushing's syndrome, and saw no effect of the drug upon the prednisone-induced impaired glucose tolerance (10); Bergenstal

et al. observed that the tolbutamide effect was abolished by ACTH infusion in an acromegalic patient (11). Creutzfeldt and Schlagintweit reported tolbutamide to have no effect on blood sugar or glycosuria in steroid diabetes resulting from administration of cortisone or prednisone (12). These difference of response are evidently not due to different doses of tolbutamide employed.

A brief comment should be made regarding the influence of diet. In controls, not receiving corticosteroids, the degree of hypoglycemia was slightly greater in animals fed the high carbohydrate diet than in those receiving stock diet, but in contradistinction to the latter the low levels were not maintained in the animal fed the high carbohydrate diet. This observation awaits confirmation on larger groups of animals. The effect of treatment with corticosteroids in abolishing or reversing the hypoglycemic effect of tolbutamide, was more marked in the animals fed the high carbohydrate diet. This is in agreement with the well known fact that the "diabetogenic" effect of corticosteroids is much more marked in rats fed a high carbohydrate diet. In our experiments there was no correlation of fasting blood sugar values, or the occurrence or degree of glycosuria during the ten days of treatment with corticosteroids, and the individual response to tolbutamide.

The mechanism by which the tolbutamide induced hypoglycemia is abolished by pretreatment with corticosteroids (this report), and intensified in the adrenalectomized animal (2, 3), is far from clear. It is quite obvious that these effects cannot be due to a decrease or an increase of "effective insulin" in the plasma, be it due to changes of insulin secretion, or of insulin degradation, or of a synergistic potentiation of insulin effects. In support of this statement, attention can be drawn to the fact that the hypophyesctomized animal does not exhibit increased hypoglycemic response to tolbutamide (2), whereas the "sensitivity" to insulin is as great as, or greater than, that of the adrenalectomized animal. Furthermore, as shown in this paper, the effect of insulin is not modified by pretreatment with cortisone in the dose employed, whereas that of tolbutamide is abolished or reversed.

Whether the action of the agents under discussion, upon glucose-6-phosphatase of the liver is the key-point, cannot at present be stated unequivocally. Cortisone treatment has been reported to increase the activity to this enzyme (13) whereas sulfonylureas exert an inhibitory action (14, 15). Such an antagonism could perhaps in part explain our observation of an antagonistic effect of corticosteroids upon the action of tolbutamide. However neither adrenalectomy nor hypophysectomy influence glucose-6-phosphatase of rat liver homogenates (16). Furthermore, Ashmore *et al.* emphasize the fact that 3 hours after administration of tolbutamide, at a time when the blood sugar is low, no effect of the drug on the enzyme could be demonstrated (15).

Altzuler *et al.* have shown that in the hypophysectomized dog, hydrocortisone increased the capacity of the liver to put glucose into the blood (17); in normal dogs Ashmore *et al.* found decreased glucose production by the liver following tolbutamide administration (18). This antagonistic action of corticosteroids, and of tolbutamide could possibly be the basis for our observations were it not for the fact that treatment with growth hormone not only did not prevent tolbutamide-induced hypoglycemia but actually intensified it. Growth hormone increases hepatic delivery of glucose (in the hypophysectomized dog) (19). The difference of the action of corticosteroids, and of growth hormone in this respect appears to be, that growth hormone, but not corticosteroids, also increases removal of blood glucose into the tissues (19); this however could hardly explain the opposite effect of pretreatment with cortisone and with growth hormone respectively, upon the hypoglycemic action of tolbutamide, reported in this paper.

Therefore no explanation of the mechanisms involved appears possible at this time.

Acknowledgments

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DEIODINATION OF THYROXINE BY HEAT-KILLED RAT KIDNEY CORTEX[1]

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ABSTRACT

When viable slices of rat kidney cortex were incubated with a thyroxine-containing medium, there was a rapid uptake of 55% of the thyroxine and production of inorganic iodide. Although similar slices heated for 10 minutes at 100° C prior to the incubation did not consume oxygen, there was still a rapid "uptake" of 50% of the added thyroxine, and considerable production of iodide.

Incubation of heat-killed kidney slices for 3 days at 5° C resulted in twice as much deiodination of added thyroxine as was carried out by living tissue under the same circumstances. When tetraiodothyroacetic acid or 3,5,3′-triiodothyronine was substituted for thyroxine, deiodination was also noted with the killed tissue, although no more extensive than with the viable.

IT HAS previously been shown that the addition of thyroxine to the medium in which slices of thyroidectomized rat kidney cortex were being incubated resulted in maintenance of oxygen consumption for three days (1). A similar effect was obtained with 3,5,3′-triiodothyronine (Trit) and 3,5,3′,5′-tetraiodothyroacetic acid (Tetrac). An extensive study of the metabolism of these three compounds has been presented (2). During the course of this work, tissue was heated for 10 minutes at 100° C to serve as controls, but at least as much deiodination was found as with the viable slices. Study of this phenomenon led to the results being reported herein.

METHODS

Slices were prepared from kidney cortex of thyroidectomized rats and incubated in Krebs' Ringer solution containing DL-alanine 0.02 *M* and glucose 0.2%, as previously described (3). Results with proline were essentially the same as with alanine, and are not reported. L-Thyroxine was added in a 0.005N NaOH-0.9% NaCl solution. Each vessel received 10 μg. per ml. plus I^{131}-tagged thyroxine to a total of 100,000–250,000 cpm. Tetrac and Trit were similarly prepared and added when desired. Tissue was killed by placing the slices in 0.9% NaCl solution exposed to a 100° C boiling water bath for 10 minutes. After being cooled to 5° C, the killed slices were rinsed with cold Ringer solution, blotted, weighed and transferred to Warburg vessels exactly as the living tissue.

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Some incubations were carried out with the conventional Warburg procedure at 37° C, and others combined daily determinations of oxygen consumption during 1.25 hours at 37° C with maintenance at 5° C during the intervening time, as used to demonstrate an *in vitro* maintenance effect of thyroxine with kidney (3). These latter experiments usually extended over 3 days. After the desired period of incubation, the contents of duplicate vessels were pooled and the medium poured off after centrifugation at 0° C. The tissue pellet was freed of medium by three rinses with saline at 0° C and was then extracted with methanol-ammonium hydroxide. Concentration of the methanol extract was carried out below 40° C under reduced pressure. Radioactivity in aliquots representing tissue and medium was counted and chromatograms made with the desired quantities. Solvents used were butanol-water-acetic acid (78:17:5) and butanol-water-ammonium hydroxide (250:178:72).

Reference spots of non-radioactive iodide, thyroxine, Tetrac, 3,5,3′-triiodothyroacetic acid (Triac) and Trit were added for qualitative identification of the peaks of activity. Spontaneous decomposition of radioactive materials sometimes occurred to an extent of 5% during incubation or manipulation of solutions without any tissue being present. It was usually less than this and appropriate corrections were always made. The values shown in the figures are expressed in terms of percentage of radioactivity present in each of the separated fractions for both tissue (bars labeled "T") and solution ("S"). Since estimations were usually made from both chromatographic solvent mixtures, the sum of tissue plus solution fractions is not always 100%.

RESULTS

Deiodination of thyroxine, Tetrac and Trit. Figure 1 is a bar-graph depicting the changes produced in these three forms of the thyroid hormone during 3 days of incubation at 5° C and 37° C (3). The live tissue, with metabolism well maintained, retained 37% of the added thyroxine as such, 13% as Tetrac plus Triac, and 2% as iodide. A further 6% was present in the medium as Tetrac to make a total of 19% metabolized by deamination and decarboxylation. Practically all of the iodide produced, 21% of 23% total, was encountered in the solution, only 2% remaining in the tissue. In the case of the killed tissue slightly more than 50% of the thyroxine was held in the tissue, corresponding closely to the amount of thyroxine plus Tetrac and Triac held by the viable slices. It is obvious that the use of the term "bound" in this situtation cannot connote an active cellular process, but it should be emphasized that the thyroxine resisted three thorough resuspension washings. The most striking finding was that 41% of the thyroxine iodine had been transformed to iodide, contrasted to 21% for the living tissue. The presence of such a large proportion of iodide was confirmed by paper electrophoresis of another aliquot. After 2 hours at 180 volts using 0.2 *M* $(NH_4)_2CO_3$ as electrolyte, 38% of the radioactivity was separated as iodide, in good agreement with the value from chromatography.

As a comparison, the experiment was repeated with an equimolar amount of Tetrac, and the results are again included in Figure 1. After the same 72-hour incubation, the viable tissue retained 45% of the added Tetrac as such, while the killed held 72%. Since the incubation solution contained

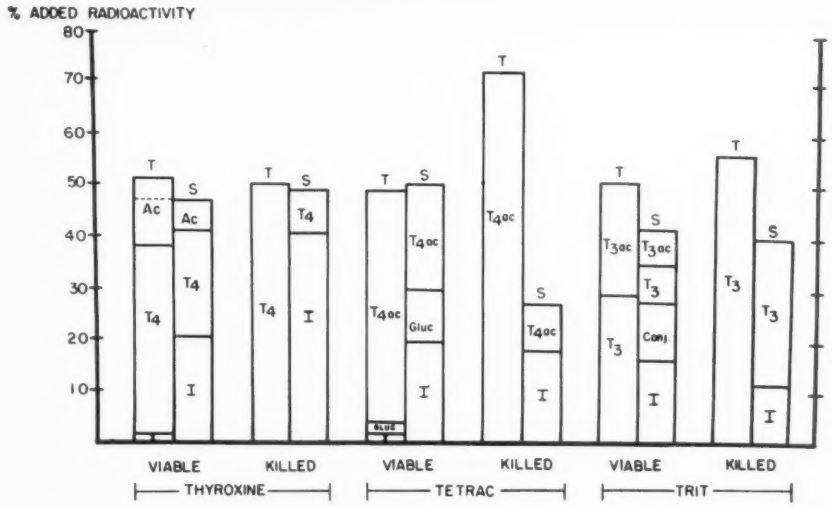

FIG. 1. Metabolism of added radioactive thyroxine, Tetrac and Trit during three-day incubation of rat kidney cortex slices in Ringer-alanine-glucose. "Killed" tissue denotes slices of viable tissue heated at 100° C for 10 minutes. Each pair of bars represents the distribution of added radioactivity between tissue ("T") and solution ("S"). Symbols: T_4, thyroxine; Ac, Tetrac plus Triac (latter above dotted line when found present); I, inorganic iodide; Gluc., glucuronic acid conjugate of Tetrac; T_4ac, Tetrac; T_3, Trit; T_3ac, Triac; Conj., glucuronic plus sulfuric acid conjugates of Trit.

9%, unchanged Tetrac totaled 81% with the killed tissue, compared to 66% for the viable. In contrast to the thyroxine incubations, the cooked slice vessels showed about the same deiodination, 18%, all in the medium, as the 22% for living tissue, most of which was outside of the slices. About 12% of the Tetrac had been conjugated by the viable kidney, primarily with glucuronic acid (cf. 2), a process completely eliminated by the heating.

When 3,5,3′-triiodothyronine was substituted in the third series, the same phenomenon of a greater retention of unchanged material by the heat-killed tissue became evident. In this case, the amount held by the killed tissues was 57%, compared to 29% as unchanged Trit in the viable slices. Since the latter also contained 22% as Triac, the tissue iodo-diphenyl-ether material amounted to 51% of the total present. Trit was deiodinated somewhat less extensively than thyroxine or Tetrac, 17% with living tissue and 12% with killed. As with Tetrac, conjugation of Trit by viable slices was a prominent metabolic feature, accounting for over 11% of the final array of substances. Two conjugates of Trit were distinguishable chromatographically, one demonstrably with glucuronic acid and the other probably with sulfuric acid.

Identification of diiodinated metabolites of thyroxine. Although not at all physiological, the considerable deiodination of thyroxine by killed tissue offered an excellent opportunity to identify the products. To this end, several experiments were carried out at 37° C for 1 to 12 hours. In addition, the glucose and alanine previously added to potentiate the thyroxine metabolic effect were omitted, to eliminate interference with various staining procedures. Extracts were made of this tissue as before, but combining contents of 6 flasks in order to obtain enough iodothyronine material for the ninhydrin stain.

From Figure 2 it is apparent that the omission of glucose and alanine re-

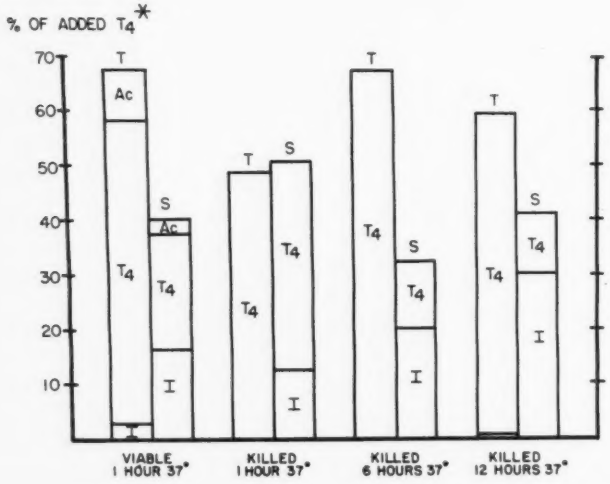

FIG. 2. Alteration of thyroxine by viable and by heat-killed kidney cortex slices incubated for varying lengths of time at 37° C. Symbols are the same as for Figure 1.

sulted in less deiodination, since even 12 hours at 37° C produced only 30% iodide, compared with the 40% previously obtained with 5 hours at 37° C plus 67 hours at 5° C. There was also 59% of the thyroxine retained as such in the cooked tissue, balanced against 50% after the longer period at 5°. Nevertheless, considerable deiodination was still obtained.

The information in Figure 2 was based on scanning for radioactivity plus the ceric sulfate-arsenious acid staining for iodine added as reference material. Even when larger aliquots of tissue extract or of medium were chromatographed for direct staining, no iodine-containing spots were encountered other than those of thyroxine and iodide as shown. Special attempts were made to detect 3,5-diiodothyronine or diiodotyrosine, without success.

Another series of chromatograms was run for staining with ninhydrin or by Pauly reagent, for amino acids and phenols, respectively. Thyroxine

was readily found in the tissue, together with other spots increasing in intensity with longer incubation. In butanol-acetic acid the R_f's were:

0.11—tyrosine in greater amounts than those found in tissue without thyroxine.

0.41—thyronine.

0.53—strongest spot, except for thyroxine; not stained by Pauly reagent.

0.73—thyroxine.

It has not been possible to identify the unknown as glycine, alanine, phenylalanine or tyrosine, the most likely possibilities.

No staining was seen in the solution, even though thyroxine was known to be present, but in inadequate amounts. Neither thyroxine nor thyronine stain well with ninhydrin or Pauly reagent, at least 25 μg. being required for each.

DISCUSSION

Deiodination of thyroxine by various tissues has been extensively studied. Since the discovery of triiodothyronine, there has been much interest in demonstrating partial deiodination to Trit as a preliminary to thyroxine activity (4, 5, 6), although contradictory results have since been obtained (7). The discrepancies in this field have not been resolved by a previous report from this laboratory (2) in which it was found impossible to identify Trit itself as a product of *in vitro* kidney cortex metabolism of thyroxine, although no other source could be found for the appreciable amounts of Triac formed from thyroxine.

Sprott and Maclagan (8) noted active deiodination of thyroxine by liver homogenates which was not lost until heating was prolonged for 2 hours at 100° C. Lissitzky *et al.* (9) found a similar deiodination of both thyroxine and Trit by homogenates of liver and pancreas, except that activity was resistant even to 2 hours' boiling. The results of Tata (10) suggest that skeletal muscle and brain homogenates carry out deiodination by a thermolabile enzyme system, since it was stated that deiodination values were corrected for a blank performed with heated material. Homogenates of kidney were still able to carry out deamination and decarboxylation, but no deiodination was seen (11). Larson *et al.* (5) have reported that kidney slices exhibited a heat-labile partial deiodination of thyroxine to Trit which was directly related to the previous level of thyroid activity in the animal. Our experiments have all been carried out on thyroidectomized rats, so no comparison with euthyroids has been made. No explanation is immediately available for the two major discrepancies with our findings of an inability to demonstrate Trit and a thermostability of complete deiodination.

Since our 10 minute period at 100° C was adequate to stop oxygen consumption, the extensive deiodination which we encountered would be considered non-enzymic in the usual sense. It can certainly be pointed out that

conjugation, deamination and decarboxylation processes were arrested. The greater deiodination occurring in the presence of alanine plus glucose and proline-glucose is intriguing, especially for a non-enzymic process. Such differences plus the strikingly high binding of thyroxine, Tetrac and Trit by the heat-killed tissue strongly suggest that the deiodination activity of this preparation is evidence of a surface-active phenomenon, and may well be quite a different process from live-tissue deiodination. Lissitzky *et al.* (9) have suggested a non-enzymic iron-containing system as being responsible for their heat-treated liver deiodination.

The lack of accumulation of appreciable amounts of partially iodinated products, such as 3,5-diiodothyronine or 3,5-diiodotyrosine, points to the remarkable efficiency of the killed tissue deiodination. A similar situation has previously been encountered in this laboratory during electrolytic desalting of organic iodine compounds (12).

Acknowledgments

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INHIBITION OF THYROIDAL IODIDE CONCENTRATING BY TRIIODOTHYRONINE: EVIDENCE FOR AN EFFECT NOT DUE TO DEPRESSION OF THYROTROPHIN BUT DEPENDENT ON THE PITUITARY[1]

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ABSTRACT

The thyroid/serum radioiodide concentration ratio (T/S) of rats receiving thiouracil (TU) was depressed to a significantly greater degree by treatment with triiodothyronine (T3) than by hypophysectomy. Differences in iodine intake did not account for this finding. T3 failed to lower the T/S (>6) of hypophysectomized rats which were given TU, whereas in unoperated rats on a TU regimen the same dose of T3 reduced the T/S to 1. It is concluded that in addition to its inhibiting effect on thyrotrophin production T3 can also interfere with thyroidal iodide concentrating by another mechanism involving the pituitary in a mediating or permissive capacity.

THYROID/SERUM radioiodide concentration ratios (T/S) lower than those usually observed in hypophysectomized rats have been repeatedly found in rats treated with large doses of thyroid-active substances (1, 2). Initially we believed that this was due to a direct action of circulating thyroid hormones on the thyroidal iodide concentrating mechanism (1). Later experiments provided no support for this concept (2). Since it was found that high iodine intake depresses the T/S in hypophysectomized rats (3), we then hypothesized (2) that the low T/S values found in animals treated with thyroid hormones were caused by abolition of thyrotrophin (TSH) secretion and a concomitant excessive iodine intake due to the increased appetite commonly seen in thyrotoxic animals.

An inverse correlation between dietary iodine levels and the T/S was seen in hypophysectomized rats receiving a standard dose of TSH. However, when organic binding of iodine in the thyroid was prevented by the administration of a goitrogen, this effect of iodine intake was no longer observed (4). This suggested that the validity of our hypothesis concerning the origin of the low T/S in thyroid hormone treated rats should be tested by comparing the effect of such hormones with that of hypophysectomy in rats on an antithyroid regimen. We also examined whether triiodothy-

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[2] Medical student summer fellow and NIH predoctoral trainee.

[3] Medical student summer fellow.

ronine, the thyroid hormone used throughout this study, had any influence on the T/S of hypophysectomized rats receiving antithyroid treatment. The findings reported below indicate that T3 can depress thyroidal iodide concentrating by a mechanism other than those hitherto considered and summarized in a recent review (5).

MATERIALS AND METHODS

Male Sprague-Dawley rats were used in this study. Intact animals weighed 200–220 gm. and hypophysectomized rats 180–200 gm. at the start of the experiments. Goitrogens given were thiouracil (TU) administered in drinking water (1 gm. dissolved in 1 liter along with 10 gm. of glucose) and, in experiment II, propylthiouracil (PTU) which was prepared as a 2% suspension and injected subcutaneously in doses of 20 mg. daily. L-3,5,3′-triiodothyronine sodium (T3) was dissolved in water made alkaline with NaOH and injected once a day subcutaneously in a volume of 1 ml. One mg. cortisone acetate in 1 ml. was also injected daily to all animals that were hypophysectomized and given TU, as well as to the rats with which these were compared. This was necessary because TU, especially in combination with T3, is poorly tolerated by hypophysectomized animals not supported with corticoids. Hypophysectomies were performed at the Hormone Assay Laboratories, Chicago, Ill. Diets fed were the Low Iodine Test Diet of Nutritional Biochemicals, Inc., Cleveland, Ohio, with or without addition of 1 or 5 mg. of iodide/Kg. (as NaI). The schedules of operations, injections and dietary regimens for the various experimental groups are presented in Table 1.

T/S values were determined according to the method of VanderLaan and Greer (6). Six mg. of PTU in alkaline solution were injected at time 0, 2–10 μc. of carrier-free I^{131} at 45 minutes, and the rats killed 1–1½ hours later. Thyroids and (in the hypophysectomized rats) testes were weighed on a Roller-Smith torsion balance. Hypophysectomized rats with grossly visible pituitary fragments and/or testes that had not atrophied to the extent appropriate for the time interval between operation and necropsy were discarded. In the hypophysectomized rats of experiment II the sella and the adjacent parts of the sphenoid and occipital bones with the attaching portions of muscles were removed *en bloc*, fixed and decalcified in Susa fluid, embedded in paraffin, sectioned serially at 10 μ, stained with hematoxylin and eosin and examined for microscopic pituitary fragments.

RESULTS

In each of the first three experiments (Table 1) rats were used that were shipped as a group and started simultaneously on the initial treatment. In Experiment I, the rats killed after 12 days on TU showed thyroid enlargement and a high T/S value (Group 1). Most of the remainder (Groups 2, 4 and 5) were hypophysectomized, others injected with T3 (Group 3). Three weeks later both hypophysectomized and T_3 treated rats (Groups 2 and 3) showed marked regression of the goiter and the T/S. The latter change was significantly greater in the rats which received T3. The T/S of the hypophysectomized rats was considerably lower than that of comparable animals in the study of Taurog *et al.* (7), probably because we did not achieve such high T/S values with our goitrogenic regimen before the operation as they did. Administration of T3 to some of the remaining hypophysectomized rats (Group 5) failed to depress the T/S significantly below the level encountered in controls not given T3 (Group 4). In experiment II an effort

TABLE 1. THYROIDAL IODIDE CONCENTRATING ABILITY OF RATS ON A GOITROGENIC REGIMEN. EFFECTS OF HYPOPHYSECTOMY, TRIIODOTHYRONINE TREATMENT AND DIETARY IODINE INTAKE

Experiment	Group	No. rats	Diet	Days fed	Goitrogen		T3		Cortisone, days given	Hypex, day	Killed, day	Testes	Thyroid	T/S	
					Drug	Days given	Dose μg./day	Days given				Wt. mg.			
I	1	6	L+1	1–12	TU	1–12	—	—	—	—	12	n.w.	37.2±2.5*	90.5±4.0	
	2	9	L+1	1–34	TU	1–34	—	—	13–34	13	34	1030±68	13.4±1.3	7.3±1.2	P<0.001
	3	10	L+1	1–34	TU	1–34	10 5	13–19 20–34	13–34	—	34	n.w.	13.5±0.5	2.2±0.2	
	4	5	L+1	1–39	TU	1–39	—	—	13–39	13	39	768±29	13.7±1.0	7.5±0.8	n.s.
	5	9	L+1	1–39	TU	1–39	2.5	35–39	13–39	13	39	737±44	12.2±0.7	6.4±0.9	
II	1	6	L	1–21	TU PTU	1–21 15–21	—	—	—	—	21	n.w.	58.8±3.7	87.7±8.7	
	2	9	L	1–39	TU PTU	1–39 15–21	—	—	22–29	22	39	1186±43	18.2±1.2	5.9±0.5	P<0.001
	3	15	L	1–39	TU PTU	1–39 15–21	10	22–39	22–29	—	39	n.w.	17.2±0.6	2.7±0.1	
III	1	12	L	7–28	TU	7–28	—	—	7–28	1	28	558±30	9.4±0.4	6.6±0.7	n.s.
	2	14	L+5	7–28	TU	7–28	—	—	7–28	1	28	572±20	10.3±0.8	6.8±1.0	
IV	1	13	L+1	8–31	TU	8–31	—	—	8–31	1	31	472±17	8.0±0.1	6.4±0.8	n.s.
	2	8	L+1	8–31	TU	8–31	5	8–31	8–31	1	31	373±12	8.2±0.4	7.7±1.0	P<0.001
	3	8	L+1	1–24	TU	1–24	5	1–24	1–24	—	24	n.w.	9.5±1.0	1.0±0.1	
	4	10	L+1	1–24	TU	1–24	—	—	1–24	—	24	n.w.	63.0±4.0	87.0±5.4	P<0.001

* Mean ± standard error.

Abbreviations: L = Low Iodine Test Diet
L+1 = same, 1 mg. iodide added per kg.
L+5 = same, 5 mg. iodide added per kg.
TU = thiouracil (0.1% in drinking water)
PTU = propylthiouracil (20 mg. day injected s.c.)
T3 = <3,5,3'-triiodothyronine sodium
Hypex = hypophysectomy performed
n.w. = not weighed
T/S = thyroid/ serum radioiodide concentration ratio
n.s. = not significantly different (P >0.1)
day 1 = the day on which the first experimental procedure was performed or started.

was made to raise the T/S more markedly by injecting PTU as well as feeding TU. However, this merely resulted in larger thyroid glands (Group 1). Again, T3 treatment was as effective as hypophysectomy in reducing thyroid size and significantly more so in depressing the T/S (compare Groups 3 and 2). Serial sections of the sellar region of the hypophysectomized rats revealed, in the majority of the cases, several small groups of epithelial cells imbedded in the scar tissue which filled the drill hole in the sphenoid bone. These cell clusters were usually discrete, but an occasional one was continuous with the pars tuberalis cells of the stalk that had become fixed to the scar. The cell groups were not penetrated by capillaries, and were composed of cells which in every respect resembled those of the pars intermedia.

Experiment III was performed to rule out a role of iodide intake on the T/S of hypophysectomized rats given TU. It became evident (compare Groups 1 and 2) that even great differences in dietary iodine levels fail to affect the T/S under such conditions.

In experiment IV, the hypophysectomized-TU treated rats in group 2 were subjected to more prolonged treatment with a higher dose of T3 than the rats in Group 5 of experiment I. Comparison with hypophysectomized rats given TU but no T3 (Group 1) showed that T3 had no depressing effect on the T/S in the absence of the pituitary. The same dose of T3 injected into normal rats (Group 3), in striking contrast, resulted in a reduction of the T/S to the lowest values ever encountered in rats not treated with anions of the thiocyanate-perchlorate class: in 4 of the 8 rats the T/S was below 1, the minimum being 0.6. Control rats not given T3 had a T/S of 87 (Group 4).

DISCUSSION

The results of this study indicate that T3, given along with TU and cortisone, is more effective than hypophysectomy in depressing the T/S of rats, whether or not they are first made goitrous with thiocarbamides. This phenomenon is not due to greater dietary iodine intake aggravating the cessation of TSH secretion in the T3 treated rats. The rats of experiment II which received T3 and showed a very low T/S could not have consumed more iodine by eating large amounts of their low iodine diet than the hypophysectomized rats in experiment III which were fed a diet of very high iodine content without consequent depression of the T/S.

It might be argued that cells producing some TSH are left behind when the hypophysis is removed, whereas T3 depresses the production of TSH in all cells. Parapharyngeal hypophysectomy indeed does not remove the cells of the pars tuberalis, and the frequent occurrence of cell groups that are apparently derived from the pars intermedia[4] and become lodged in

[4] Displaced pars intermedia cells would be expected to "take" more readily than those of the pars distalis, since the pars intermedia shows hypertrophic-hyperplastic changes in response to disruption of hypothalamo-hypophysial connections (8, 9).

the scar tissue of the drill hole further emphasizes that complete ablation of even the subdiaphragmatic adenohypophysial tissue may not be customarily achieved. However, there is no evidence for the production of TSH by either pars tuberalis or pars intermedia cells. Further, if hypophysectomized rats showed residual TSH production, T3 would be expected to abolish it, whereas we found that T3 exerts no effect on the T/S of hypophysectomized rats.

There is no doubt that T3, like any other thyroid-active substance, inhibits thyroid functions (including the concentrating of iodide) by interfering with the production of TSH. The findings reported in this paper necessitate the assumption that T3 has an additional effect which is either mediated through the hypophysis or depends on the permissive action of one or more pituitary or target gland principles.

Reforzo-Membrives (10) has asserted that the pituitary of thyroid-fed rats contains a substance which upon injection into guinea pigs reduces thyroid weight and follicular cell height and lowers the BMR. His findings have not been confirmed and doubt has been cast on the adequacy of this experimental procedures (11). Pending comfirmation of his observations by others an examination of their relation to our findings does not seem worth while.

All rats in this study were receiving TU and the doses of T3 used were undoubtedly pharmacological. It is therefore questionable whether the previously unrecognized thyroid-inhibiting action of T3 described in this paper plays any role in physiological thyroid regulation. It also remains to be shown whether this effect of T3 is shared by other thyroid-active substances. If such is the case, the drastic reduction of the T/S previously achieved with thyroxine, tetrabromothyronine and tribromothyronine (2) might have involved the same mechanism.

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THE INHIBITION OF THE METABOLISM OF THIOCYANATE IN THE THYROID OF THE RAT[1]

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ABSTRACT

There is no *concentration gradient* for thiocyanate ion between the thyroid and serum. However, thiocyanate ion is metabolized by the thyroid with sulfate being the major sulfur product. This metabolism is completely inhibited by thiourea, sodium iodide, and sulfadiazine, but is only slightly inhibited by sodium perchlorate.

IT HAS been reported that the thiocyanate ion is metabolized by the thyroid and that the sulfur atom is oxidized in part to sulfate (1). Further interest in this reaction has been stimulated by subsequent observations which revealed that the thiocarbamide-type anti-thyroid compounds, such as thiourea (2, 3), thiouracil (3), and 4-methylthiouracil (4), are also metabolized by the thyroid, their sulfur atom being oxidized to sulfate. The thyroid would seem, therefore, to possess an effective mechanism for desulfurating and oxidizing the sulfur of these anti-thyroid compounds. The object of the present study is to explore further the nature of the metabolism of thiocyanate by the thyroid and to investigate the effect of certain inhibitors of thyroid function on this reaction.

EXPERIMENTAL

Materials.—S^{35}-thiocyanate and S^{35}-thiourea were obtained from the New England Nuclear Corporation (Boston, Massachusetts); the specific activities ranged from 10.0 to 43.0 millicuries per millimole. The purity of the compounds was confirmed by paper chromatography.

Animals.—Male Sprague-Dawley rats weighing 100–175 gm. were maintained on Purina Fox Chow pellets.[2] Animals of nearly the same age and weight were used in a single experiment, since the metabolism of thiocyanate by the thyroid varied significantly when these factors were not controlled.

Analytical Methods.—The S^{35}-thiocyanate was administered intraperitoneally as a single dose, 20 micromoles (μmoles). At intervals, the animals were killed by ether and exsanguination. The thyroid and a portion of liver were removed, cleaned, weighed

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[2] Iodine content, 2.0 to 3.0 μg./gm. food.

(wet weight), and then homogenized in 0.5 ml. chilled triple-distilled water. For comparison, the homogenization was also carried out with ethylene-diaminetetraäcetate (0.001 *M*); no difference was noted in the final results. The blood was centrifuged and the serum removed. Appropriate aliquots of the tissue homogenate, the serum, and S^{35} standards were assayed for total S^{35}-radioactivity by methods previously described (3).

The S^{35}-metabolites were characterized by paper chromatography. A ten to forty microliter aliquot of the tissue homogenate or serum and 0.2 micromole each of thiocyanate and sulfate carriers were applied along a 1.5 cm. line to Whatman No. 1 chromatography paper. Chromatograms were run in an ascending solvents system of ethanol-1 *M* ammonium acetate, pH 7.5 (7.5:3). The radioactive spots along the paper strips were located by autoradiography using No-Screen x-ray film. The carrier spots were identified as follows: thiocyanate by spraying with 10% ferric chloride and sulfate by spraying with 0.024% barium chloride followed by 0.024% potassium rhodizonate. A typical chromatogram is shown in Figure 1. The amount of protein-bound sulfar, sulfate, and of thiocyanate present was determined by cutting up the chromatogram, by counting the radioactivity in each area, and by expressing it as a percentage of the total number of counts along the strip.

Effect of various compounds on the metabolism of thiocyanate by the thyroid.—Solutions of thiourea, sodium iodide, sodium sulfadiazine, and sodium perchlorate were administered intraperitoneally fifteen minutes prior to the administration of the S^{35}-thiocyanate, 20 μmoles. The animals were then sacrificed one hour following the administration of thiocyanate and the tissue analyses for radioactivity carried out as outlined above.

FIG. 1. Radioautogram of a paper chromatogram run in an ascending solvent system of ethanol-1.0 *M* ammonium acetate, pH 7.5 (7.5:3). This solvent separates sulfate with an Rf of 0.24 and thiocyanate with an Rf of 0.91. Radioactivity remaining at the origin is presumed to be protein-bound sulfur (PBS^{35}).

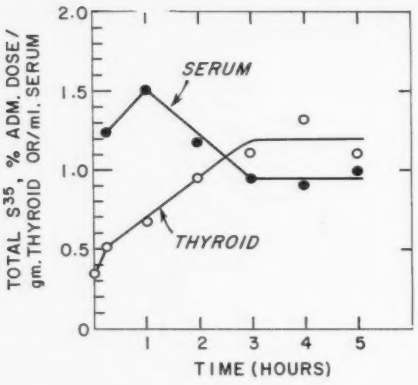

FIG. 2. The per cent of total S^{35}-radioactivity found per gram of thyroid and per ml. of serum following the intraperitoneal administration of a single dose of 20 μmoles of S^{35}-thiocyanate to each of a group of normal rats.

All experiments were performed at least two or three times, each on a different day. Each table represents a typical experiment with the data at each point being derived from a single animal.

RESULTS

Following the intraperitoneal administration of a single dose of 20 μmoles of S^{35}-thiocyanate to each of a group of rats, there is an increase in the total S^{35}-radioactivity in the thyroid over a 3–4 hour period (Fig. 2). The nature of the S^{35}-radioactivity in the thyroid, liver, and serum, as determined by paper chromatography, is shown in Table 1. Differences are readily apparent; in five hours, only 26.0% of the total S^{35}-radioactivity in the thyroid is thiocyanate, in contrast to values of 95.0% in the serum, and 99.0% in the liver. In one experiment, data was obtained over a ten-hour period. At the end of six, eight, and ten hours, there was 17.0%, 13.0%, and 11.0%, respectively, of the total S^{35}-radioactivity in the thyroid as thiocyanate. 96% of the total radioactivity in the serum was identified as thiocyanate after ten hours.

TABLE 1. PAPER CHROMATOGRAPHY OF THE S^{35}-RADIOACTIVITY IN THE THYROID, LIVER, AND SERUM AFTER THE ADMINISTRATION OF 20 μMOLES OF S^{35}-THIOCYANATE TO RATS

	Interval after administration of thiocyanate	S^{35} spots, % of total S^{35} along the paper chromatogram		
		Origin (PBS^{35})	Sulfate	Thiocyanate
Thyroid	5 min.	0.3	5.0	92.0
	15 min.	0.0	14.0	79.0
	1 hr.	0.0	37.0	59.0
	3 hrs.	1.6	60.0	38.0
	5 hrs.	3.2	69.0	26.0
Serum	1 hr.	—	—	97.0
	5 hrs.	—	1.0	95.0
Liver	1 hr.	—	—	99.0
	5 hrs.	—	—	99.0

TABLE 2. CONCENTRATION OF THIOCYANATE IN THE THYROID AND SERUM FOLLOWING THE ADMINISTRATION OF 20 μMOLES OF S^{35}-THIOCYANATE TO RATS

Interval after administration of thiocyanate	Concentration of thiocyanate in serum,* mμmoles/ml.	Concentration of thiocyanate in thyroid,* mμmoles/gm.
5 min.	—	64
15 min.	238	82
1 hr.	286	82
2 hrs.	230	69
3 hrs.	186	77
4 hrs.	180	77
5 hrs.	184	57

* Calculations from experimental data of Fig. 2 and Table 1.

Concentration of thiocyanate in the thyroid and serum.—The concentration of thiocyanate in the thyroid is fairly constant, 57 to 82 millimicromoles /gm. (mμmoles/gm.), during the five-hour period following its administration (Table 2). In this interval the concentration of thiocyanate in the serum ranged from 180 to 286 mμmoles/ml. Clearly, there is no *concentration gradient* for thiocyanate between the thyroid and serum, the ratio (T/S) varying from 0.29 to 0.34.

S^{35}-metabolic products.—It was determined that the thyroid metabolizes thiocyanate at a maximum rate of about 65 mμmoles/gm. thyroid/hour. The major sulfur product of this metabolism is sulfate (Table 1), which is identified by paper chromatography (Fig. 1) and by paper electrophoresis (Fig. 3). Sulfate has the same Rf as sulfite and thiosulfate in the paper chromatography system but was distinguished from these two compounds by paper electrophoresis. Only a trace of protein-bound sulfur, 2–3% of the

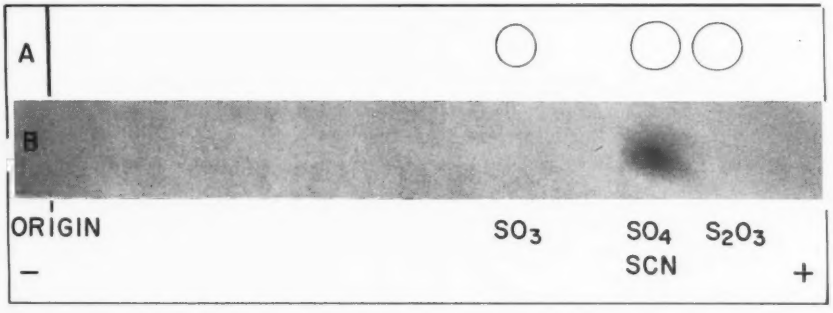

FIG. 3. Paper electrophoresis (A) and its autoradiogram (B). Paper electrophoresis using thin Whatman No. 31 paper, Tris citrate buffer (0.06 *M*-0.03 *M*), ionic strength 0.18, pH 7.0, 1000 volts for 35 minutes (5). This method separates sulfite with a mobility of 15.5 cm., sulfate and thiocyanate with a mobility of 20.0 cm., and thiosulfate with a mobility of 22.0 cm., under a potential gradient of 17.0 V/cm. Sulfite and thiosulfate are identified by spraying the paper with 0.1 N alcoholic iodine, thiocyanate by spraying with 10% ferric chloride. Thiocyanate has the same mobility as sulfate in this system, but is easily separated from it by paper chromatography (Fig. 1).

TABLE 3. INHIBITION BY THIOUREA OF THE METABOLISM OF THIOCYANATE IN THE RAT THYROID

	Rats	Dose thiourea adm., μmoles	Concentration of thiocyanate in thyroid, mμmoles/gm.	Thiocyanate metabolized by thyroid, mμmoles/gm.
1	Control		130	45.0
2	Control		130	40.0
3		5	150	10.0
4		10	220	8.0
5		20*	200	3.0

Conditions as in the Experimental section.
* The approximate concentration of thiourea in the thyroid is 110 mμmoles/gm.

total radioactivity, is found in the thyroid (Table 1), even at intervals up to 10 hours after the administration of thiocyanate. This value for the amount of PBS^{35} formed is lower than that previously reported (1). Increasing the administered dose of thiocyanate to levels as high as those used by Wood and Williams (1), namely 264 μmoles, failed to produce any increase in the quantity of protein-bound sulfur.

In an attempt to obtain further information concerning the metabolism of thiocyanate, it was decided to investigate the effect of certain representative anti-thyroid compounds on this reaction.

Thiourea.—This thiocarbamide-type anti-thyroid compound, a potent inhibitor of the iodination reaction in the thyroid (6), produces a 93% inhibition of the metabolism of thiocyanate when equimolar doses of both compounds are administered to rats (Table 3). At the same time, thiourea seems to produce an increase in the concentration of thiocyanate in the thyroid.

Since the sulfur of thiourea is also oxidized to sulfate by the rat thyroid (2, 3), it seemed of interest at this point to reverse the above experiment. As noted in Table 4, thiocyanate does not inhibit the metabolism of thiourea when equimolar doses are administered. However, doses of thiocyanate 4 and 8 times those of thiourea do produce approximately a

TABLE 4. INHIBITION BY THIOCYANATE OF THE METABOLISM OF THIOUREA IN THE RAT THYROID

	Rats	Dose thiocyanate administered, μmoles	Concentration of thiourea in thyroid, mμmoles/gm.	Thiourea metabolized by thyroid, mμmoles/gm.
1	Control		110	67.0
2	Control		110	77.0
3		10	120	87.0
4		20*	100	68.0
5		40	160	77.0
6		80	150	40.0
7		160	130	38.0

The animals were given non-radioactive thiocyanate, followed in 15 minutes by 20 μmoles of S^{35}-thiourea, then killed in one hour. Paper chromatography of thiourea as previously described (3).
* The approximate concentration of thiocyanate in the thyroid, 150 mμmoles/gm.

TABLE 5. INHIBITION BY SODIUM IODIDE OF THE METABOLISM OF THIOCYANATE IN THE RAT THYROID

	Rats	Dose iodide adm., μmoles	Conc. thiocyanate in thyroid, mμmoles/gm.	Thiocyanate metabolized by thyroid, mμmoles/gm.
1	Control		150	40
2	Control		154	40
3		0.1	104	40
4		1.0	130	16
5		5.0	129	3
6		20.0	90	1
7		100.0	149	3

Conditions as in Experimental section..

45% inhibition. It is noteworthy that thiocyanate does not modify the concentration of thiourea in the thyroid.

Sodium Iodide.—The effect of this halide was studied, since it not only inhibits the iodination reaction (7) but is a known antagonist to thiocyanate in thyroid physiology (8). As shown in Table 5, iodide is a potent inhibitor of the metabolism of thiocyanate. Further observations suggest that the inhibitory effect of a constant dose of iodide is only partially overcome by increasing the dose of thiocyanate.

Sodium Sulfadiazine.—Sulfadiazine, an aromatic-type antithyroid compound which inhibits the iodination of tyrosyl groups in the thyroid (9) also inhibits the metabolism of thiocyanate (Table 6). At the same time, this compound, unlike thiourea and iodide, appears to produce a significant decrease in the concentration of thiocyanate in the thyroid.

Sodium Perchlorate.—This compound does not inhibit the iodination reaction (10, 11), but is a very potent inhibitor of the transport of iodide into the thyroid (12). As noted in Table 6, perchlorate is a poor inhibitor of the metabolism of thiocyanate.

DISCUSSION

Thiocyanate ion inhibits the transport of iodide into the thyroid (13, 14), yet there is no *concentration gradient* for it between the thyroid and

TABLE 6. INHIBITION BY SODIUM SULFADIAZINE AND SODIUM PERCHLORATE OF THE METABOLISM OF THIOCYANATE IN THE RAT THYROID

	Dose of inhibitor, μmoles	Conc. of thiocyanate in thyroid, mμmoles/gm.	Thiocyanate metabolized by thyroid, mμmoles/gm.
Control		226	65.0
Control		244	65.0
Sulfadiazine	37	189	3.0
	185	123	1.0
Perchlorate	20	244	63.0
	40	248	70.0
	80	154	40.0

Conditions as in the Experimental section.

serum. This observation is in accord with that of Wood and Williams (1) and Wollman *et al.* (15), but differs from that of Logothetopoulos and Myant (16) which revealed a *concentration gradient* of two to five/one. This figure is still much lower than that for the *concentration gradient* of iodide between thyroid and serum.

Thiocyanate ion is metabolized by the thyroid of the rat at a maximum rate of about 65 mμmoles/gm. thyroid/hour. Sulfate is the major sulfur product. Propylthiouracil, which inhibits the formation of protein-bound iodine in the thyroid (17), also inhibits the metabolism of thiocyanate (1). Furthermore, thiourea, iodide, and sulfadiazine also produce this dual inhibition. However, the metabolism of thiocyanate is not inhibited by comparable doses of sodium perchlorate, a compound which presumably affects only the concentration of iodide by the thyroid. Hence, it is possible that a common mechanism may be involved in the iodination of tyrosyl groups and the metabolism of thiocyanate in the thyroid.

Of the three anti-thyroid compounds which inhibited the metabolism of thiocyanate in this study, only thiourea produced an actual increase in the level of thiocyanate in the thyroid. This is of interest since thiocyanate ion was found to be a specific requirement for the *in vitro* desulfuration of thiourea by cytoplasmic particulate fractions of sheep thyroid (18).

Thiocyanate is a ubiquitous anion in the body, presumably being formed as a product of the "detoxification" of cyanide (19–24). The serum level of thiocyanate in the normal rat is reported to be about 55 mμmoles/ml. (25). The present study revealed that following the intraperitoneal administration of 20 μmoles of thiocyanate to rats, there was a maximal inhibition of the concentration of iodide by the thyroid,[3] at a time when the serum level of thiocyanate ranged from 180 to 286 mμmoles/ml. (Table 2). Hence, it would appear that there is a narrow range between the level of thiocyanate normally in the serum of the rat and the level at which there is an inhibition of the concentration of iodide by the thyroid. The role of thiocyanate ion in the homeostasis of the thyroid remains a provocative question.

Acknowledgments

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THE NATURE OF THE SEXUAL DIMORPHISM OF THE SUBMANDIBULAR GLAND OF THE MOUSE[1]

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ABSTRACT

The weights of the submandibular glands and their water, lipid and non-lipid fractions were determined in intact, gonadectomized and androgen-treated gonadectomized mice of both sexes. It was found that although the glands are markedly heavier in intact males than in females, the composition of the tissue is the same in both sexes. The loss of weight after gonadectomy is accounted for solely as loss of water in the female, whereas in the male there is loss both of water and dry tissue residue. Treatment with androgen results in the increase in the absolute amount of water and dry tissue residue in both sexes. The present data indicate that the larger size of the glands in intact male and androgen-treated mice is due primarily to differential growth of the terminal secretory ducts.

HAMMETT (1) discovered that the submandibular gland is signifi cantly larger in male than in female rats, and Lacassagne (2) and Fekete and Ossen (3) reported that in mice there is a sexual dimorphism in the structure of the terminal secretory tubules of the gland. Subsequently the testes (4), hypophysis (5), ovaries (6), adrenal cortex (7) and thyroid (8) have all been shown to influence the cytology of these tubules. Harvey (9) has demonstrated that in mice during sexual maturation and following gonadectomy and androgen treatment the weights of the glands change in a manner parallel to the changes in histologic structure.

Despite the abundant research on the submandibular glands in rodents, the question remains concerning the basic mechanism responsible for the greater glandular mass in intact males and androgen-treated animals of both sexes. That is, is the greater size due to actual growth of the secretory tubules or to apparent growth simulated, for instance, by an increase in tissue water content under the influence of the male sex hormone? The present experiment was designed to resolve this question.

EXPERIMENTAL

Seventy-eight 3 to 4 month old SWR mice (approximately equal numbers of males and females) were used. The mice were divided into 6 groups of 11 to 15 animals each

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as follows: (1) untreated intact males; (2) males gonadectomized for 10 to 12 days; (3) males gonadectomized for 10 to 12 days, then injected subcutaneously with 2.5 mg. of testosterone propionate in oil on 4 alternate days; (4) untreated intact females; (5) females gonadectomized for 10 to 12 days; (6) females gonadectomized for 10 to 12 days, then injected subcutaneously with 2.5 mg. of testosterone propionate in oil on 4 alternate days. The mice were killed with chloroform (2 days after the final hormone injection in the case of those receiving treatment) and were weighed. The submandibular glands were removed, cleansed of adherent tissue and weighed to the nearest tenth of a milligram on a Roller-Smith torsion blance. The glands were then dried to a constant weight under an infrared lamp for 3 days. After the weight was recorded, the dried tissues were extracted with 3 one-day changes of a mixture of equal parts of ethanol, chloroform and ether to remove the free lipids. Thereafter, the glands were dried again and the final weights recorded.

RESULTS

The results are summarized in Table 1. The statistical significance of the data has been examined by the analysis of variance.

Wet weight of the glands

1. The effect of sex in intact mice

   The glands are significantly heavier in males than in females (Group 1 vs. Group 4, $P = 0.001$).

2. The effect of gonadectomy

   Removal of the gonads results in a significant decrease in the weight of the glands in both males and females (Group 1 vs. Group 2 and

TABLE 1. WEIGHTS OF SUBMANDIBULAR GLANDS IN INTACT, GONADECTOMIZED AND ANDROGEN-TREATED GONADECTOMIZED MICE

Group	Treatment	No. of mice	Submandibular glands			
			Total wet wt. (mg./25 gm. body wt.)	Water (mg.)	Lipid (mg.)	Non-lipid (mg.)
1	Intact males	11	207.0	167.0 80.7%	4.1 2.0%	35.8 17.3%
2	Gonadect. males[1]	11	137.8	107.0 77.6%	3.6 2.6%	27.3 19.8%
3	Androgen-treated[2] gonadect. males	15	194.1	145.0 74.7%	4.7 2.4%	44.4 22.9%
4	Intact females	15	161.2	131.4 81.5%	3.1 1.9%	26.7 16.6%
5	Gonadect. females[1]	13	123.3	94.5 76.7%	3.2 2.6%	25.6 20.7%
6	Androgen-treated[2] gonadect. females	13	181.0	136.3 75.3%	4.0 2.2%	40.7 22.5%

[1] Gonadectomized for 10 to 12 days.
[2] Gonadectomized for 10 to 12 days, then injected with 2.5 mg. of testosterone propionate on 4 alternate days.

Group 4 vs. group 5, $P=0.001$ in each instance). The final weights reached by the glands after gonadectomy are of questionable difference in the two sexes (Group 2 vs. Group 5, $P=0.02$).

3. Effect of androgen in gonadectomized mice

   Treatment of castrated males results in the return of the weight of the glands to a level not significantly different from that in the intact male controls (Group 3 vs. Group 1, $P=0.1$). Treatment of ovariectomized females, on the other hand, produces an increase in the weight of the glands to a level that is significantly higher than that in the intact female controls (Group 6 vs. Group 4, $P=0.01$), but which is significantly lower than that in intact males (Group 6 vs. Group 1, $P=0.001$).

Lipid Fractions of the Glands

There was no significant difference in the size of the lipid fractions of the glands in any of the groups of mice studied.

Water and Non-lipid Fractions of the Glands

1. The effect of sex

   There was no significant difference between intact males and females in the sizes of the water and non-lipid fractions of the glands (Group 1 vs. Group 4). Likewise there was no significant difference between males and females in the composition of the glands after gonadectomy (Group 2 vs. Group 5) or after subsequent androgen treatment (Group 3 vs. Group 6).

2. The effect of gonadectomy

   In both sexes there was a significant decrease in the water and increase in the non-lipid fraction (Groups 2 and 5 vs. Groups 1 and 4, $P=0.001$). It is interesting to note, however, that in females this change is due to the loss of water alone, the absolute size of the non-lipid fraction remaining unchanged. In males, on the other hand, there is a reduction in the absolute sizes of both the water and non-lipid fractions of the glands after gonadectomy.

3. The effect of androgen in gonadectomized mice

   In both sexes there was further significant decrease in the water and non-lipid fractions as compared with the untreated gonadectomized animals (Groups 3 and 6 vs. Groups 2 and 5, $P=0.001$). It is also interesting to note that in contrast to the situation following gonadectomy, after androgen treatment there is an increase in both the water and non-lipid fractions of the glands of both males and females.

COMMENT

It seems clear from the present observations that since the wet weights of the submandibular glands are significantly different in intact male and

female mice, and since the relative sizes of the water, lipid and non-lipid fractions of the tissues are the same, the basic factor accounting for the sexual dimorphism is that of an absolute difference in the tissue masses of the glands in the two sexes due primarily to the differential growth of the tubular elements. The possibility that the larger size of the gland in the male might be due, for instance, to a greater content of water relative to lipid and non-lipid has been eliminated. It is also evident from the present data that the decrease in the weight of the glands after gonadectomy is due to different mechanisms in the male and the female. In the latter the weight reduction is accounted for solely by loss of water from the tissue, there being no appreciable change in the absolute weight of the dry residue. The response of the glands to androgen treatment, on the other hand, is similar in gonadectomized animals of both sexes. That is, the absolute size of both the water and dry tissue fractions is increased subsequent to hormone administration, indicating tissue growth has taken place.

The decrease in the relative water content of the glands of both sexes subsequent to gonadectomy and again after androgen-treatment is interesting but its cause remains obscure. It is possible that the values obtained may represent but transient stages in the regression and regrowth of the glandular tissue. This question cannot be settled from the data on hand since only one time interval after gonadectomy and after the onset of hormone treatment was utilized in the design of the experiment.

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INTESTINAL ABSORPTION OF INSULIN IN THE RAT

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ABSTRACT

The intestinal absorption of insulin in the rat has been investigated. Amorphous zinc-free insulin and proteolytic inhibitors (soybean trypsin inhibitor and diisopropylfluorophosphate) were injected into ligated sections of jejunum and absorption of insulin measured by the concomitant depression of blood glucose. The effect on insulin absorption of previously injected indole-3-acetate was also determined.

Diisopropylfluorophosphate (DFP) is effective as a depressant of insulin digestion and biologically active insulin is absorbed in small quantities in the presence of this inhibitor. Soybean trypsin inhibitor is not effective. Intraperoneal injection of indole-3-acetate one hour previous to the intestinal administration of insulin and DFP enhances the hypoglycemic action of absorbed insulin.

Using an everted sac technique, insulin was shown to pass through the intestinal wall. This was demonstrated by chromatographing the sac contents and by the hypoglycemic action of the sac contents when injected into normal rats.

A NUMBER of recent investigations have indicated that intact proteins can be absorbed from the small intestine of mammals providing intestinally-contained proteases are sufficiently inhibited. One of the proteins most frequently employed in attempts to demonstrate intestinal absorption of protein is insulin, since the assay of the appearance in the blood stream is facilitated by a concomitant lowering of blood glucose. Jensen (1) has reviewed earlier attempts to administer insulin by means other than intravenous or intramuscular injection. Laskowski et al. (2) have demonstrated a reduction in blood sugar in rats following simultaneous intestinal administration of insulin and a pancreatic trypsin inhibitor. Their results present strong evidence for the absorption of intact insulin, although not positive proof, since the site of action and size of the physiologically active molecule are still unknown.

The present communication is a report of experiments in which insulin and proteolytic enzyme inhibitors (other than pancreatic trypsin inhibitor) were introduced into the intestines of living normal and depancreatized rats; and also, experiments using a modified Wilson and Wiseman (3) everted sac technique in which insulin served as the mobile factor. These latter studies lend conclusive evidence that the insulin molecule absorbed is in fact the same as the molecule presented for absorption. Experiments

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were also conducted wherein indole-3-acetate (IAA) was given to rats prior to the intestinal administration of insulin, since it had been reported (4, 5) that this compound increases the activity of insulin, probably by inhibiting a liver insulinase.

MATERIALS AND METHODS

Insulin. An important consideration in intestinal protein absorption studies is the dimensions of the molecule presented for absorption. Hallas-Moler (6) has shown that different insulin preparations contain different particle sizes, and in view of further evidence (7) concerning the forces causing aggregation of insulin molecules, it was decided to use a zinc-free amorphous insulin[1] in these experiments.

Soybean trypsin inhibitor. This inhibitor has been shown to react mole for mole with trypsin *in vitro* (8) forming a stable complex and thus inhibiting trypsin. Wu (9) has shown that this anti-enzyme also has some inhibitory effect on beta chymotrypsin and, to a slight degree, on the alpha form of chymotrypsin. The soybean trypsin inhibitor used in this work was prepared according to the method of Kunitz (10).

Diisopropylfluorophosphate (DFP)[2] has been shown to destroy the proteolytic and esterolytic activities of both trypsin and all forms of chymotrypsin when used in very small quantities (11).

Female Sprague-Dawley rats weighing 200–250 gm. were starved for 20 hours and then anesthetized with nembutal (40 mg./kg. body weight). A front midline incision was made exposing the viscera. A ligature was tied around the jejunum approximately 20 cm. from its juncture with the duodenum. A hypodermic needle, attached to a syringe containing the solution to be tested, was then carefully inserted into the lumen of the jejunum at its union with the duodenum. A ligature was tightened about the section of intestine containing the needle and the contents of the syringe slowly introduced into the intestine. The needle was withdrawn from the lumen while at the same time the ligature was tightened securely about the jejunum. At measured time intervals thereafter, blood samples (0.1 ml.) were obtained by clipping the end of the tail. Glucose concentration of these samples was determined by Nelson's method (12). The solutions introduced in this manner consisted of insulin and insulin plus proteolytic enzyme inhibitors, and the appropriate controls for such experiments.

In the everted sac technique, 250 gm. female Sprague-Dawley rats were killed by a blow on the head and the abdomen opened by a midline incision. While intact, the entire small intestine was washed with a solution of 0.9% NaCl containing 0.3% glucose. The entire small intestine was removed and the mesentery manually stripped from it. A glass rod was used to push the ileal end of the intestine into its lumen, and the entire length of the intestine thus everted, placing the mucosal lining on the outside and the serosal layer on the inside. The everted intestine was placed in a glucose-saline solution at room temperature. Double ligatures were tied around one end of 2–3 cm. lengths of the jejunum. A blunt hypodermic needle attached to a one ml. syringe was used to fill the sac from the open end, adding a bubble of air, and then securing ligatures. The sacs were placed in individual 25 ml. flasks containing the solutions being tested for absorption and incubated for one hour at 37°. Contents of the sac as well as the incubating solution were assayed for appropriate constituents.

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[1] The insulin used was generously supplied by Dr. Otto K. Behrens, Eli Lilly and Co., Indianapolis, Indiana.

[2] The DFP used in these experiments was obtained from the Aldrich Chemical Company, Milwaukee, Wisconsin.

RESULTS AND DISCUSSION

Introduction into rat intestine of massive quantities of insulin and crystalline soybean trypsin inhibitor in amounts sufficient to inhibit two or more times the maximal quantity of trypsin in the intestine resulted in no blood glucose drop, or very small and nonuniform responses. This was the case in normal and also depancreatized rats (Table 1). In only one case (that of a depancreatized rat) was there a significant insulin effect with soybean inhibitor. Insulin alone has no effect when administered via the intestinal lumen.

Preliminary experiments indicated that 0.075 mg. DFP could be administered into the intestinal lumen of rats without a detrimental effect and that such treatment alone produced no blood glucose lowering. This level of DFP (0.075 mg. dose) was used in all of the experiments herein reported. When insulin was introduced into the intestine along with the DFP there was a marked and consistant blood glucose depression (Table 1). This was the case in the presence or absence of concomitant soybean trypsin inhibitor and although experiments were not specifically designed to prove the point, it was considered that the soybean inhibitor did not enhance the DFP effect. In the presence of DFP, 240 USP units of insulin/Kg. had a blood glucose depressing effect on normal rats; 160 USP units were not effective. In depancreatized rats 160 USP units of insulin/Kg. had a blood glucose depressing effect, but 80 units/Kg. were ineffective

TABLE 1. INTESTINAL INJECTION OF INSULIN AND PROTEOLYTIC INHIBITORS

Rats		Insulin, USP units/Kg.	Soybean trypsin inhibitor mg.	DFP, mg.	Minutes after insulin						
N	D*				0	30	60	90	120	150	180
					Blood glucose (mg%)						
+		800	—	—	80	81	80	80	81	80	79
+		800	100	—	65	70	70	65	70	65	65
+		800	200	—	67	67	69	70	68	69	70
+		—	100	—	65	67	65	65	67	68	—
	+	1600	—	—	190	195	190	—	205	200	210
	+	1600	100	—	210	200	190	200	190	185	193
	+	1600	100	—	235	109	89	expired			
	+	1600	100	—	210	205	198	195	208	210	200
	+	—	100	—	200	200	210	200	200	—	210
+		—	—	0.075	60	60	62	60	62	60	60
+		800	—	0.075	60	55	19	expired			
+		240	—	0.075	75	80	60	49	45	41	38
+		160	—	0.075	76	81	70	70	75	74	75
+		80	—	0.075	81	81	81	80	79	81	81
+		240	150	0.075	78	76	62	50	48	40	—
	+	—	—	0.075	185	190	177	—	177	180	—
	+	400	—	0.075	135	115	110	90	50	expired	
	+	160	—	0.075	210	190	180	120	90	70	70
	+	80	—	0.075	200	195	197	200	185	177	195
	+	40	—	0.075	173	177	167	165	178	172	170
	+	400	100	0.075	130	90	80	expired			

* N-Normal.
D-Depancreatized.

when used with DFP. The extent of absorption of intraintestinally administered insulin in the experiments described is thus estimated to be of the order of one two-thousandth of the amount given, since intravenous injection of about one tenth unit per kilogram of the insulin used caused a substantial blood glucose lowering in similar fasted normal rats; one twentieth unit was effective in depancreatized rats.

Experiments in which 3mM/Kg. of IAA[3] was injected intraperitoneally one hour previous to the intraintestinal injection of insulin and DFP indicated that the IAA "protected" the absorbed insulin and permitted it to be more effective in reducing blood glucose (Table 2). This was the case in both normal and depancreatized rats. IAA alone or IAA plus intestinal administration of insulin without DFP were ineffective in reducing blood glucose.

An experiment in which the IAA was administered two and a half hours following a minimal intraintestinal dose of insulin with DFP and this in turn followed one and a half hours later by a second intestinal administration of half the initial dose of insulin with DFP, is illustrated in Figure 1. It appears that the role of IAA in this experiment is not the usually considered "protective" one, but rather that the IAA is allowing a lower level of insulin to reduce blood glucose.

Using the everted sac technique, sections of rat small intestine were incubated with shaking for one hour at 37° in Krebs phosphate solution with insulin or insulin plus DFP. At the beginning of the incubation period the contents of the sac consisted of 0.5 ml. of added Krebs phosphate solution. Following the incubation period the contents of the sac were chromat-

TABLE 2. THE EFFECT OF INDOLE-3-ACETATE (IAA) ON INTESTINALLY ABSORBED INSULIN

Rats		Insulin, USP units/kg.	DFP mg.	IAA 1 hr. Previous, 3mM/kg.	Minutes after insulin						
N	D*				0	30	60	90	120	150	180
					Blood glucose (mg.%)						
+		—	—	+	65	64	62	65	66	65	66
+		800	—	+	69	70	65	63	64	67	68
+		240	0.075	−	75	80	60	49	45	41	38
+		240	0.075	+	81	81	69	57	46	21	10
+		160	0.075	−	76	81	70	70	75	74	75
+		160	0.075	+	75	75	61	50	39	36	39
+		80	0.075	−	81	81	81	80	79	81	81
+		80	0.075	+	84	85	81	76	61	55	55
+		40	0.075	+	77	78	70	72	65	71	5
	+	400	0.075	−	135	115	110	90	50	expired	
	+	400	0.075	+	140	50	expired				
	+	160	0.075	−	210	190	180	120	90	70	70
	+	160	0.075	+	181	175	143	121	96	42	expired
	+	40	0.075	−	173	177	167	165	178	172	170
	+	40	0.075	+	130	135	120	107	100	90	82
	+	24	0.075	+	150	150	145	153	147	150	147

* N—Normal.
D-Depancreatized.

[3] Indole-3-acetate was obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio.

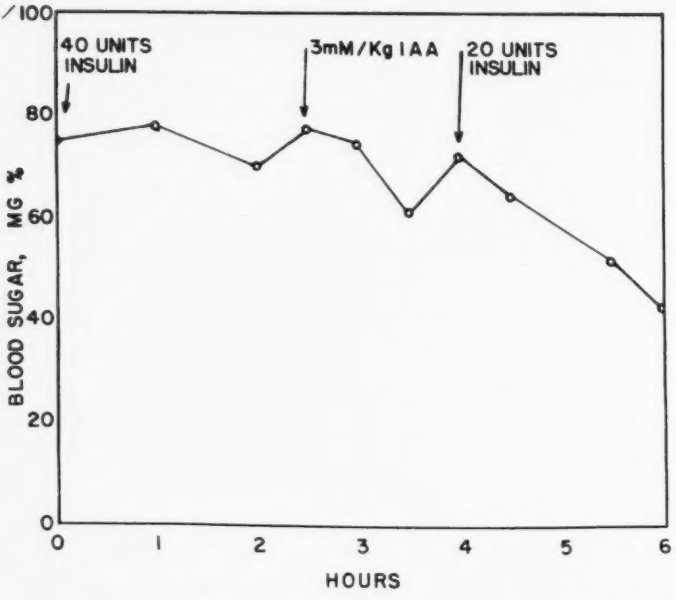

FIG. 1. The effect of indole-3-acetate on the hypoglycemic action of intestinally administered insulin. 160 units/Kg. of insulin and 0.075 mg. DFP were given intraintestinally to normal rats at zero time; 3 mM/Kg. of IAA were injected intraperitoneally 2.5 hrs. later; and 80 units/Kg. of insulin with 0.075 mg. DFP given intraintestinally 4 hrs. from the beginning of the experiment.

ographed on paper for insulin according to the method of Grodsky (13). Control chromatography experiments of the insulin used gave R_F values of 0.21. The contents of the sac in the absorption experiments showed a strong spot chromatographically at R_F 0.21. This was the case in both the presence and absence of DFP. The failure of the proteolytic inhibitor to show an effect on the absorption of the insulin in this procedure is readily attributed to the washing of the mucosa prior to the incubation period, thus eliminating the proteolytic enzymes.

In a similar series of experiments using the everted sac, the contents of the sac and two small saline washings of the interior of the sac were recovered and diluted to one ml. (It was estimated that the contents of the sac occupied a volume of about 0.5 ml. following incubation.) A one-tenth ml. aliquot of the diluted sac contents was intravenously injected into normal rats and blood samples taken for glucose assay. In all cases where insulin (100 U.S.P. units/ml.) was present in the incubating medium, the 10% aliquot of sac contents at the end of incubation caused a marked reduction (30 to 50 mg. %) in blood glucose content. Control experiments without insulin gave no change in blood glucose when sac contents were injected

into normal rats. On the basis of this hypoglycemic response to sac contents it can be roughly estimated that the insulin concentration inside the sac at the end of incubation was approximately 2% that of the incubating medium.

Intraintestinal administration of insulin plus a potent proteolytic enzyme inhibitor such as DFP permits the insulin to enter the circulating blood and reduce blood glucose. In the absence of a proteolytic inhibitor the results of such experiments are negative indicating that the administered insulin is destroyed in the gut.

In *in vitro* experiments using everted sacs of rat intestine, insulin can be shown to cross the intestinal lining and appear within the sac as indicated by its position during paper chromatography and by the ability of the sac contents to reduce blood glucose upon intravenous injection into normal rats.

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THE RESPONSE OF OVARIAN EXPLANTS FROM POST-NATAL MICE TO GONADOTROPINS

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ABSTRACT

The morphological changes that occur in ovarian explants from young mice maintained in an *in vitro* system for a period of 7 days in the presence of several gonadotropins were studied. Although the explants cultured on the basic medium composed of agar, Tyrode's solution, synthetic medium 199 and chick embryo extract and enriched with lactogenic hormone retained a healthy appearance, histological examination revealed a general loss of organization with selective atrophy of the follicular and of the thecal cells. As a consequence, maintenance of the primordial follicles was not obtained and their maturation was arrested. Some or all of these degenerative changes did not occur when other hormones were added to the basic medium. Either ICSH, HCG or estrogen effected stimulation of the follicular cells and, at the same time, favored maturation of the primordial follicles; in addition, ICSH effected an organization and a stimulation of the theca interna. PMSG induced changes that were not unlike those observed in the hypophysectomized rat. In every instance, certain of the changes produced with the gonadotropins could be produced with the steroid hormones alone, and the effects obtained under *in vitro* conditions were comparable to those previously observed under *in vivo* conditions.

GONADOTROPIN preparations stimulate gonads of intact and hypophysectomized animals. Three distinct activities have been identified: follicle-stimulating hormone (FSH) promotes growth of the testes and of the ovarian follicles (1, 2); interstitial cell-stimulating hormone (ICSH) and human chorionic gonadotropin (HCG) affect the non-germinal tissue of the gonads in both sexes and stimulate spermatogenesis in the male (1, 3, 4, 5); lactogenic hormone exhibits luteal and mammotropic functions (6, 7). Pregnant mare serum gonadotropin (PMSG) possesses both FSH-like and ICSH-like activities (8, 9).

Although the gonadotropins are effective in the absence of the pituitary gland, it remains to be demonstrated that these hormones also can act independently of other endocrine tissue. In an abstract, Barnhart (10) reported that the oxygen uptake of ovaries of newborn mice was enhanced in the presence of FSH; other attempts to effect stimulation of the explanted ovary have yielded only negative results (11, 12). The work reported here demonstrates that ICSH, HCG and PMSG do stimulate,

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under *in vitro* conditions, consistent morphological changes in ovaries taken from post-natal mice.

EXPERIMENTAL PROCEDURE

Female mice of the C57 Black strain and of an inbred white strain reared in the local colony were used for the study. Ovaries were taken 4 days after birth and explanted to a medium made up of 2 parts agar,[2] 2 parts Tyrode's solution enriched with an equal quantity of synthetic medium 199 (13), and 1 part chick embryo extract (1 volume chick embryos: 1 volume Tyrode's solution); the cultures were carried in embryological watch glasses. General details of the method have been described previously (14). Operations were carried out with sterile materials in an isolated room. Systematically, the flat surface of the ovary that contained ova but no developing follicles was placed in contact with the medium. Prior to the time the tissues were placed in an incubator maintained at $37 \pm 1°$ C, the liquid that had accumulated around the explants was absorbed with sterile filter paper and the embryological watch glasses were sealed with paraffin. At 24-hour intervals the explants were displaced to a new site on the medium and, midway through the culture period or at $3\frac{1}{2}$ days, they were transferred to fresh media following a bath with Tyrode's solution. The same procedure was followed each time that an explant was transferred from one receptacle to another; 0.4–0.5 cc. of Tyrode's solution was added to the watch glass containing the explant, the explant was gently dislodged from the surface of the medium, and the organ was then transferred to the new medium in a small volume of Tyrode's solution with a pipette. After 7 days of culture, the explants were fixed in Bouin's solution, embedded in paraffin, and sectioned serially at 6 micra. Subsequently, they were examined for histological changes.

The ovine lactogenic hormone (30–35 I.U./mg.) was prepared by Dr. C. H. Li (15); the ovine ICSH was prepared by Dr. P. G. Squire (16); the HCG (1000 I.U./mg.) was a product of Leo Pharmaceutical Co.; the PMSG (15,000 I.U./mg.) was prepared by Dr. J. Legault-Démare (17).[3] These gonadotropins were dissolved directly in the enriched Tyrode's solution; on the other hand, the estrogen and progesterone were first dissolved in 100% ethanol and then diluted 20-fold with the Tyrode-medium 199 solution. For the preparation of the culture medium, 0.2 cc. of the hormone solution was added to a small bottle contaiuing 0.2 cc of warm 1% agar and 0.1 cc. of chick embryo extract; the contents were mixed with a glass stirring rod and the preparation immediately drawn into a tuberculin syringe. 0.05 cc. of the hormone-enriched medium was then added to each embryological watch glass in which a mixture of 6 drops agar, 6 drops Tyrode's solution and 3 drops extract had already congealed. Quantitative measurements were made with 1 cc. tuberculin syringes, and glassware used in the preparation of the medium was warmed before agar was introduced in order to avoid premature gelation of the preparation. It is essential that the operation be completed quickly if the preparation of a medium having a proper consistency is to be assured.

RESULTS

Females of the albino strain were used exclusively for the studies with the gonadotropins inasmuch as there was a marked difference in the de-

[2] An autoclaved agar preparation containing 1 gm. agar (Bacto)/100 cc. Gey's solution. This preparation remained fluid and homogeneous when heated in a water bath at 80° C.

[3] The author wishes to express appreciation to the 4 sources listed above for hormones used in this study.

gree of ovarian differentiation attained by the 2 strains of mice at a given age. For example, by the 4th day after birth the ovaries of the white mice contained many ovocytes surrounded by abundant cytoplasm and numerous primary follicles made up of a single row of flattened follicular cells encircled by thecal cells (Fig. 1A). In contrast, the ovaries of the C57 mice contained some primitive ova with granular cytoplasm, few or no primary follicles, and showed no thecal organization (Fig. 1B).

Changes with cultivation. Ovaries taken from post-natal mice showed morphological changes when cultured in an *in vitro* system for a period of 7 days. The histological sections from the ovaries of the albino mice suggest a general loss of organization; the non-germinal cells are less conspicuous and the ovocytes have come to occupy a major portion of the ovary (Fig. 1C). Closer examination reveals that the cytoplasm in the follicular cells is reduced and that thecal cells are no longer in evidence. No advancement in the development of the primary follicles and no increase in their number was ever observed (Fig. 1D).

Changes with hormones. Although most of the changes observed in the ovary after 7 days of culture can be described as atrophic, they cannot be considered as necrotic. Figures 2C, 2F and 2G illustrate that several hormones exhibited a capacity to prevent some or all of the disorganization described above. Among the gonadotropins, only lactogenic hormone was ineffective in this respect; contrariwise, 200 I.U. of HCG and 0.040 mg. of ISCH/0.1 cc. of the medium maintained the general structure of the ovary, stimulated the thecal cells, and maintained the follicular cells. 0.0002 mg. of estradiol, like 0.040 mg. of ICSH, not only maintained the follicular cells but also induced their hyperplasia as can be seen in Figures 2F and 2G. PMSG at a dosage level of 200 I.U. stimulated the stromal tissue but at the same time promoted degenerative changes in the larger primordial follicles; these phenomena included atresia of the ova and a peculiar destruction of the follicular cells (Fig. 2D).

DISCUSSION

The development of the ovary in the young mouse is not a spontaneous process. Although the intermediary determinants responsible for sexual orientation and differentiation in the embryonic stage have not been identified, without a doubt the ultimate influence must rest with genetic factors. The sex chromosome in the male is different from that in the female and, as demonstrated here, the degree of ovarian differentiation evident shortly after birth varies markedly with the strain of mouse studied. A further indication that the process is not spontaneous is derived from *in vitro* studies with the isolated gonad. In a most thorough investigation Martinovitch (18) demonstrated that whereas growth and differentiation of the germ cells occur in 100% of the cultures, growth of the follicles is partly and sometimes completely suppressed. At the same time he pointed out that

the culture medium composed of chicken plasma and chick embryo extract, although most likely free of gonadotropic hormones, probably contained sex hormones. More recently, Foote and Foote (19) have observed that

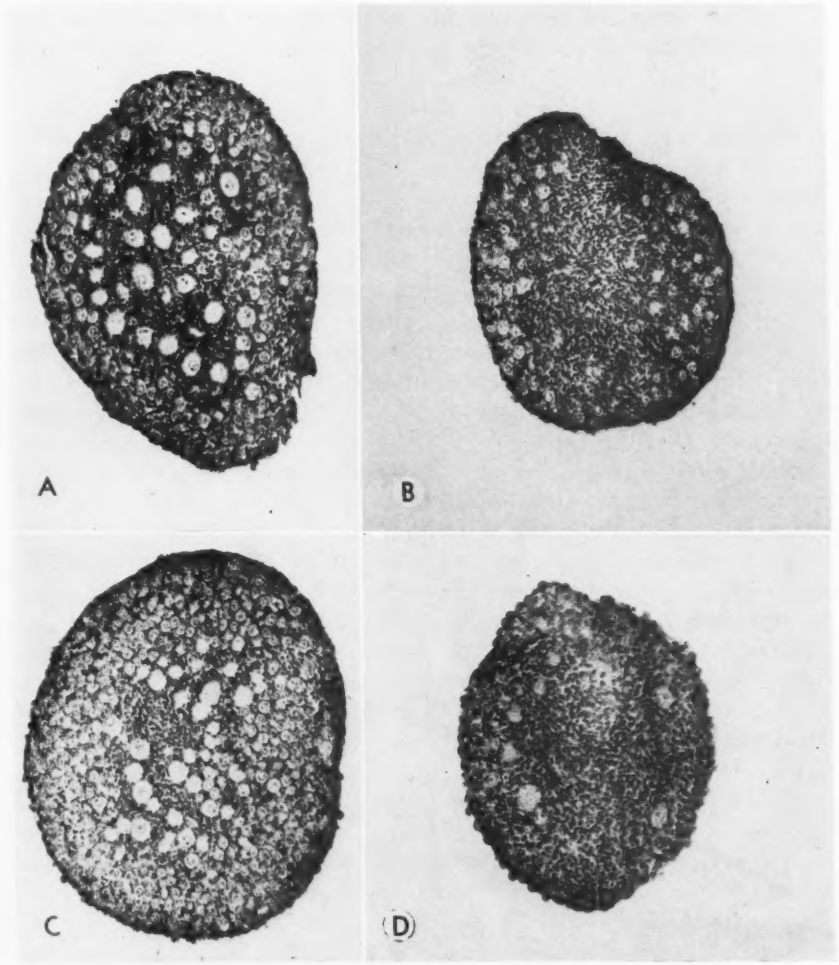

FIG. 1. Sections of gonads from female mice, 4th day after birth, 6 micra in thickness. Hematoxylin-eosin stain ×200.

A. Ovary of normal albino mouse. Numerous primary follicles already present.

B. Ovary of normal C57 Black mouse.

C. Ovary of albino mouse after 7 days of culture on basic medium enriched with lactogenic hormone. Ovocytes more in evidence but no advancement in follicular development.

D. Ovary of C57 mouse after 7 days of culture on the basic medium. No further maturation.

there is more cellular outgrowth from ovarian explants cultured on a medium composed of cock plasma and embryo extract than on one composed of agar and embryo extract. It is also possible that the presence of steroids in plasma may account for the remarkable follicular development obtained by the Italian workers (20) which prompted them to conclude that isolated gonads continue to evolve in the same manner and at the same rate as if they were left to develop in the animal.

In the present experiments, the isolated ovary has been shown to respond to gonadotropic and to steroid hormones. Not only was the atrophy of the follicular and thecal cells prevented, but their stimulation was actually effected. As a consequence, the organization of the ovary was maintained and the growth of the follicles proceeded as in the whole animal. The action of each hormone was specific and its activity *in vitro* corresponded to its activity in the animal body. Thus, ICSH and HCG induced similar morphological changes at the doses employed. Moreover, the specificity of ICSH lay in its capacity to organize the theca interna. There is other histological evidence that the theca plays an important role in follicular development and ovulation, as discussed by Hisaw (22). From the present study it is evident that the effects of ICSH on the primary follicle, other than stimulation of the theca, were comparable to those observed with estradiol. It therefore seems likely that ICSH was able to negotiate these changes by virtue of its capacity to stimulate the production of estrogen. As to whether or not the cells of the theca interna themselves are capable of secreting the estrogen that in turn stimulates the proliferation of the follicular cells and the formation of a stratum granulosum remains to be determined.

The relationship of estrogen to the development of the primordial follicle was first demonstrated by Pencharz (23) when he observed that the ovaries of immature hypophysectomized rats given diethylstilbestrol consisted of medium-sized follicles tightly packed together. This observation has been confirmed by a number of investigators, and Simpson, *et al*, (24), have shown that whereas stilbestrol and FSH act synergistically, estrogen and ICSH do not. The current *in vitro* studies reemphasize the importance of the steroid hormones in the maturation of the follicles and their ova, and they likewise suggest that ICSH and estrogen are essential for the develop-

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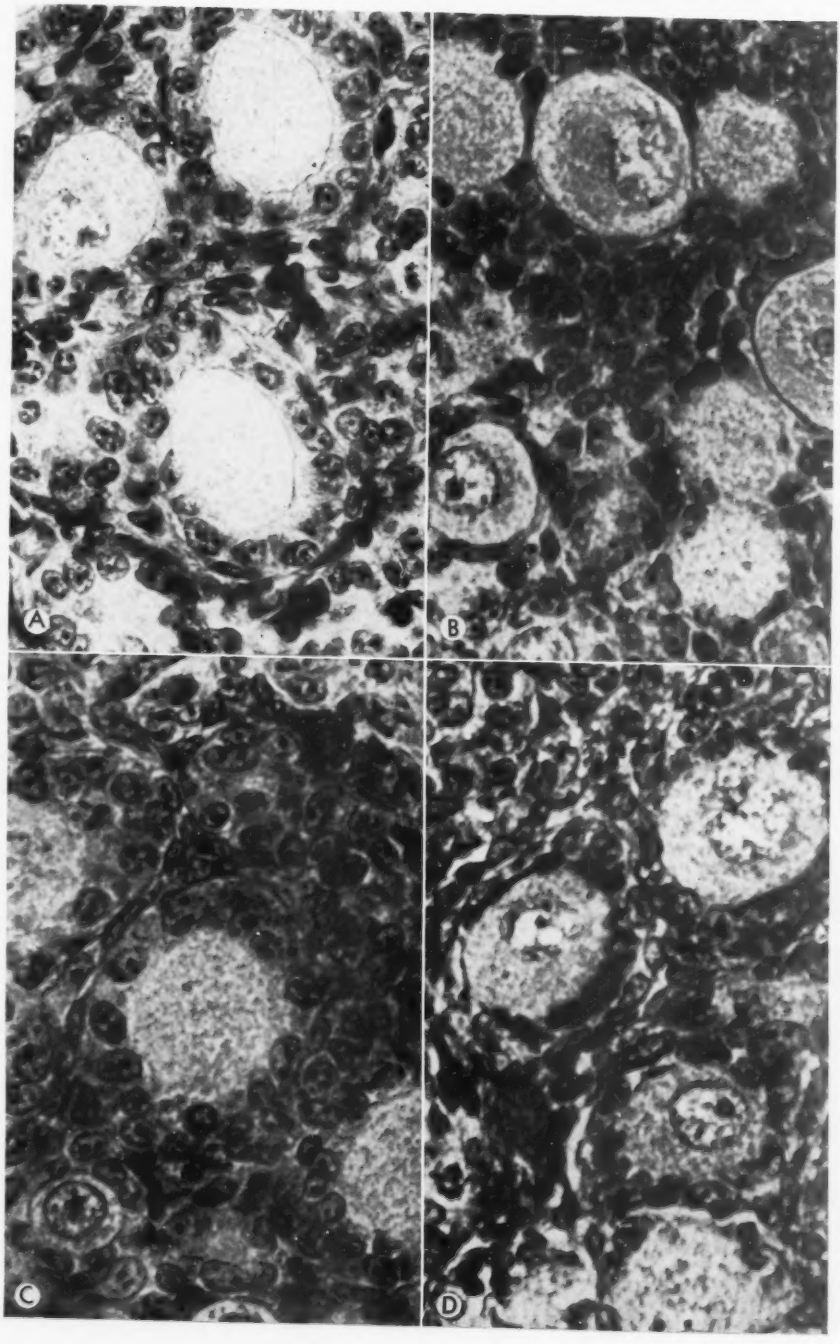
FIG. 2. Detailed structure of ovaries taken from mice, 4th day after birth, and cultured on hormone-enriched media for a period of 7 days. Sections 6 micra in thickness, hematoxylin-eosin stain ×1320.

A. Albino mouse, no culture.

B. Albino mouse, medium enriched with lactogenic hormone, 0.20 mg./0.1 cc. Note atrophy of follicular and thecal cells resulting in a general loss of organization.

C. Albino mouse, medium enriched with HCG, 200 I.U./0.1 cc.

D. Albino mouse, medium enriched with PMSG, 125 I.U./0.1 cc. Peculiar degeneration of the follicular cells in the larger follicles.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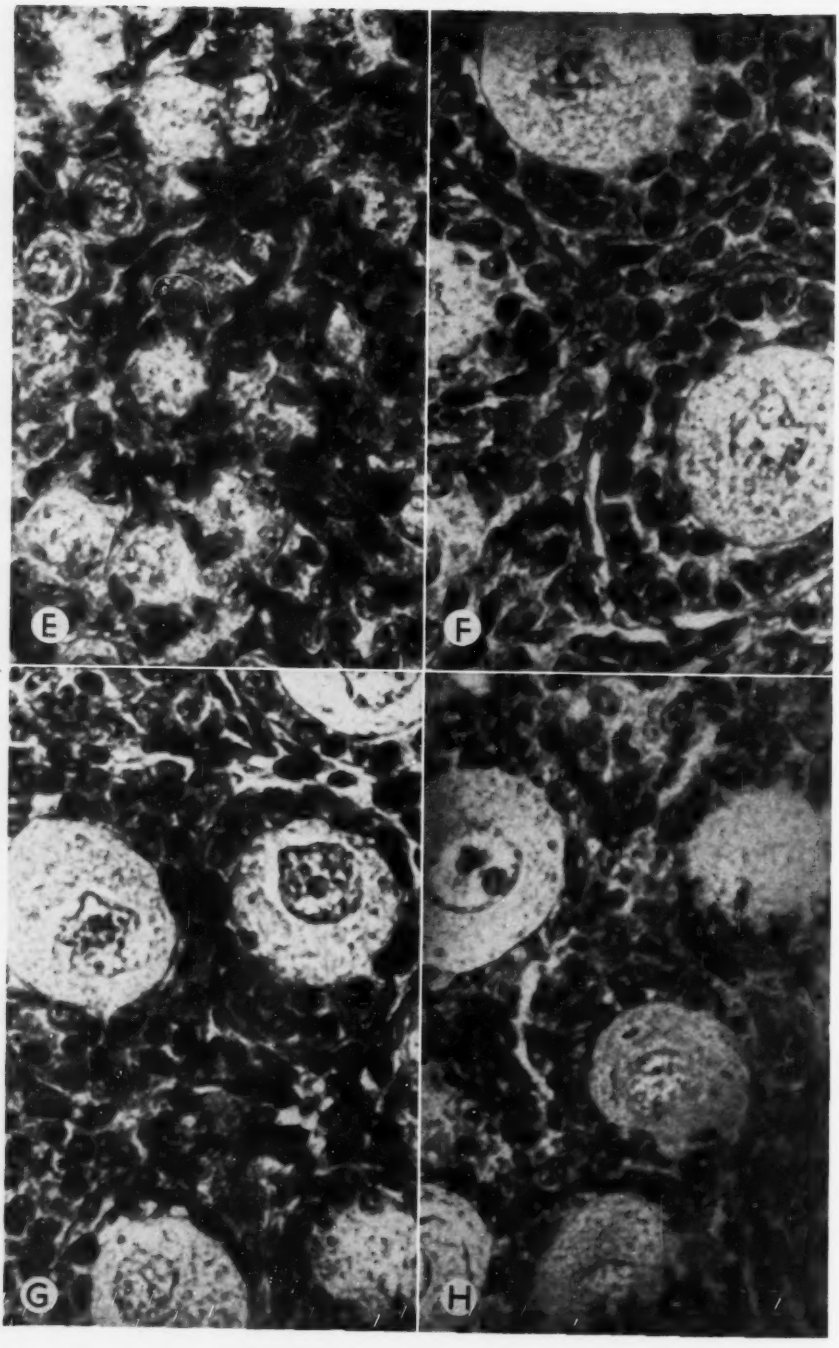
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ment of the mammalian ovary at an earlier stage than has been envisioned previously.

The morphological changes induced with PMSG were different from those observed with ICSH; moreover, they corresponded to the effects obtained in female rats when large doses of the gonadotropin are injected immediately after hypophysectomy (25). These results are not aberrant inasmuch as it has been shown previously that highly-purified PMSG fractions display FSH-like, ICSH-like and luteinizing activities (9); the preparation of PMSG used in this investigation likewise exhibited the 3 gonadotropic activities mentioned above (17). In this instance what is of interest is that ovaries from young female mice respond in a comparable fashion to the same hormones under *in vitro* conditions as do those of hypophysectomized immature rats under *in vivo* conditions.

The *in vitro* method presented here lends itself to definitive biological studies; it is applicable both to qualitative and to quantitative determinations. However, as described, it is not useful for microdeterminations inasmuch as it is no more sensitive than is the assay employing the hypophysectomized animal. The efficiency of the system undoubtedly could be increased if the hormone solution were supplied directly to the explant rather than incorporated into a small quantity of the basic medium. In these experiments, the injection technique was purposely avoided since certain hazards are associated with it; injury to the cells can result if the solution is injected into the organ and destruction of the cells can occur if liquid accumulates around an explant (26).

Acknowledgment

I wish to express my sincere appreciation to Professor Etienne Wolff and the members of his laboratory for a most pleasant and rewarding year in the Lab. D'Embryologie Exptl. where the organ culture method was acquired and adapted to these hormonal studies.

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Fig. 2—(*Continued*)

E. C57 mouse, no culture.

F. Albino mouse, medium enriched with ICSH, 0.040 mg./0.1 cc. Organization of theca interna and stimulation of follicular cells.

G. Albino mouse, medium enriched with estradiol benzoate, 0.0002 mg./0.1 cc. Stimulation of follicular cells.

H. Albino mouse, medium enriched with progesterone, 0.002 mg./0.1 cc.

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NOTES AND COMMENTS

EFFECTS OF HYPOPHYSECTOMY ON SOME FUNCTIONAL ASPECTS OF THE ADRENAL GLAND OF THE CHICKEN*

ABSTRACT

There was no statistically significant change in gross adrenal gland weight, or change in concentration of ascorbic acid, cholesterol, or Δ^4-3-ketocorticosteroids in the adrenal glands of hypophysectomized birds compared to control birds. Hypophysectomy was followed by a 50% reduction in blood acidophils. These results imply a great degree of functional independence of adrenal cortical tissue from the anterior pituitary in the chicken.

INTRODUCTION

Reports of the effect of hypophysectomy on the structure and/or function of the adrenal of birds have varied (1–6). The evidence used in evaluating adrenal function following hypophysectomy has been gross adrenal weight (1–4), adrenal cytology (3, 5), and physiologic information of an indirect nature (6). In view of the uncertainty that the anterior pituitary is essential for "normal" function of the bird's adrenal cortical tissue, it is necessary to study this relationship by more direct means.

METHODS

Hypophysectomized and normal White Leghorn cockerels of corresponding age were purchased from the Endocrine Laboratories, Madison, Wisconsin. Hypophysectomy was performed when the birds were 63–81 days of age. Data from only those operated birds which showed a marked regression of comb and testicular weight at autopsy were used in this study.

At the various times indicated in Table 1, the birds were killed by decapitation and the following determinations made by slight modifications of the respective procedures: adrenal cholesterol (7), adrenal ascorbic acid (8), and the adrenal Δ^4-3-ketocorticosteroids (9, 10). Blood acidophil counts were made by a procedure already described (11).

RESULTS

Results of the various determinations performed to study possible functional aspects of adrenal cortical function in hypophysectomized birds are listed in Table 1. The hypophysectomized chickens showed increased deposition of body fat and in this manner maintained, in most groups, body weights approaching those of the controls (4).

Hypophysectomy did not result in a significant gross weight change of the birds' adrenal glands under the conditions of time used in this study. Neither was there a statistically significant change ($P < .05$) in the concentration of ascorbic acid or cholesterol in the adrenal glands from the hypophysectomized birds as compared with those from the controls. The reason for the marked difference in concentration of free steroids in the controls of groups 1 and 3 is unknown; however, the glands of group 3 were stored in a frozen state for 5 months in contrast to 1 month for those of group 1.

Since injection of ACTH has been found to be followed by an acute acidophilia, which has some merit as a criterion of stress in the chicken (12), the effect of chronic hypophysectomy on the acidophil count was studied. Results of counts made at two intervals following operation are shown in Table 2. A decrease in acidophils by about 50% was evident four weeks following hypophysectomy.

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TABLE 1. EFFECT OF HYPOPHYSECTOMY ON VARIOUS ADRENAL GLAND CONSTITUENTS

Group	Treatment	Days post-operative	Body weight§	Adrenal weight*	Ascorbic acid†	Cholesterol‡	Free corticosteroids†
1	Control		(8) 1695	(8) 7.86±0.5	(7) 50.43±2.9	(7) 4.32±0.9	(7) 7.10±1.7
	Operated	25–29	(8) 1411	(8) 9.42±3.2	(7) 50.58±5.7	(7) 3.43±1.0	(7) 8.70±3.6
2	Control		(5) 1114	(5) 10.62±1.9			
	Operated	26	(5) 757	(5) 12.08±2.6			
3	Control		(5) 1415	(5) 10.44±1.9			(5) 2.10±0.8
	Operated	36	(5) 1184	(5) 9.76±8.4			(5) 2.00±1.6
4	Control		(4) 1574	(4) 9.12±2.2	(4) 74.78±5.7	(4) 4.92±1.1	
	Operated	58	(4) 1466	(4) 8.11±1.8	(4) 58.98±2.2	(4) 4.52±1.0	

The number in parenthesis indicates number of animals.
* mg./100 gm. body weight.
† μg./100 mg. tissue
‡ mg./100 mg. tissue.
§ gm.

DISCUSSION

The lack of substantial decrease in adrenal weight following hypophysectomy in the present study is in contrast to the decrease obtained by others using chicks and pigeons. Schooley, Riddle and Bates (2) reported at 27% decrease in adrenal weight following hypophysectomy of pigeons; however, recalculation of their figures on the basis of body weight reduces this figure to about 8% (no additional statistical information is available). In addition, these workers indicated that while there was histological evidence of atrophy of cortical elements there was also evidence of hypertrophy of cortical tissue at the periphery of the cortical strands. A somewhat similar situation was reported by Nalbandov and Card (5) for the adrenal of the hypophysectomized chicken. They found an "almost complete initial degeneration with a possible subsequent partial recovery."

Although the concentrations of adrenal cholesterol in the operated animals were reduced by 8% (group 4) and 20% (group 1), the differences did not approach statistical significance (P was $<.5$ and $<.1$ respectively). The absence of a marked change in concentration of adrenal cholesterol following hypophysectomy is in agreement with histochemical evidence in hypophysectomized pigeons (3). This evidence, together with that of the ascorbic acid determinations in the hypophysectomized birds is in accord with previously published findings that injection of ACTH into birds does not consistently cause a change in either adrenal cholesterol or ascorbic acid (13–18). It is generally believed that adrenal cholesterol is the precursor of adrenal corticosteroids; the functional significance of adrenal ascorbic acid is in doubt.

A marked acidophilia has been found to be an indicator of the injection of ACTH or the application of any of a number of stressors in the chicken (11, 12). It was shown that the magnitude of acidophilia under similar circumstances was curtailed but not entirely prevented by hypophysectomy (11). The present indication that hypophysectomy alone produces an acidopenia over a period of four weeks (Table 2) suggests that the

TABLE 2. BLOOD ACIDOPHIL COUNTS

Days postoperative	Hypophysectomized	Controls	P*
15	(11) 27.4 ±14.9	(7) 34.68±11.7	$<.2$
28	(10) 17.45± 8.	(7) 38.28±17.5	$<.01$

Figures represent (number of birds) mean number of acidophils ± standard deviation per 0.09 mm.³ blood.
* Probability based on Student's "t" test.

failure to obtain maximal acidophilia might have been due to an inability of the hematopoietic elements to respond rather than a failure of an endocrine mechanism to mobilize. It is to be noted, however, that the exact mechanism by which stressors cause an acidophilia in the chicken (11) is not known.

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ADRENALINE CONCENTRATION IN THE RAT UTERUS AFTER ADRENLINE ADMINISTRATION BY DIFFERENT ROUTES[1]

ABSTRACT

Experiments were performed to determine why subcutaneous injections of adrenaline did not cause a decrease in glycogen and phosphorylase levels in the rat uterus while intraperitoneal injections did. Intraperitoneal injection of small amounts of adrenaline was followed by a large increase in the uterine level of adrenaline (chemically determined) 10 minutes later, in spayed rats pretreated with estradiol. Subcutaneous injection of similar or larger doses of adrenaline was not followed by an increase in uterine adrenaline levels in 10 minutes. The uterine adrenaline levels, one hour after 100 μg. doses injected subcutaneously, were above the control level but similar to those found one hour after 25 μg. doses, given intraperitoneally. This indicated that a threshold level of adrenaline must be attained rapidly to produce these metabolic effects. Uterine noradrenaline levels were increased one hour after injecting 100 μg. doses of adrenaline subcutaneously.

Adrenaline administered intraperitoneally, causes a decrease in the glycogen level (1) and the amount of active phosphorylase (2) in the estrous rat uterus but does not have

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this effect if given subcutaneously even in large doses. One possible explanation for the failure of subcutaneously injected adrenaline to produce these effects might be that it reaches the uterus in insufficient amounts. This explanation would be substantiated if it could be shown that a much higher level of adrenaline is present in the uterus after an intraperitoneal injection than after a similar dose injected subcutaneously. In this investigation, adrenaline and noradrenaline levels in the uterus were measured following injections of adrenaline by the two different routes.

METHODS

Adrenaline and noradrenaline levels in the uteri were determined fluorometrically by the Weil-Malherbe method as adapted to the Farrand Fluorometer (3). This method permits the determination of both catechol amines simultaneously. Uterine glycogen was determined by methods previously described (1).

Adult female rats (200–250 gm.) were spayed, and 6–8 days later all were injected with 50 μg. of estradiol subcutaneously. Pretreatment with estrogen served to physiologically "standardize" the uterine tissue, to produce sufficient amounts of tissue from one rat for analysis and to facilitate homogenization. 48 hours after estrogen treatment, L-adrenaline-HCl (Parke Davis Co.) was injected in different doses, per 100 gm. body weight. While the rats were anesthetized with ether, their uteri were removed 10 to 60 minutes after adrenaline injections, trimmed and emptied of fluid and frozen between 2 blocks of dry ice. The uteri were weighed, then homogenized in cold 10% trichloroacetic acid and the hormones extracted as described for skeletal muscle (3). Data are reported as μg. of hormone per gm. of tissue.

RESULTS

Intraperiteoneal injections of 50 μg. of adrenaline which was shown previously to lower both glycogen and phosphorylase when given by this route, was followed by a rise in the uterine adrenaline level from 0.02 to 3.04 μg. per gm. of tissue within 10 minutes but no noradrenaline was found. Previous work has shown that excessive amounts of adrenaline relative to noradrenaline (4) or *vice versa* (3) in a mixture of the two will not permit accurate measurement of the hormone present in the lesser quantity. Since it was desired to determine noradrenaline levels simultaneously, optimal doses of adrenaline to circumvent this difficulty were sought. Intraperitoneal doses of 25 μg. adrenaline permitted the subsequent determination of both catechol amines, and also decreased the uterine glycogen levels by 33% within 10 minutes (from control values of 240 ± 17 mg.% to 161 ± 9 mg.%; 12 rats).

The data in Table 1 show that when 25 μg. of adrenaline were given intraperitoneally, the adrenaline concentration in the uterus increased within 10 minutes and was considerably above the initial level at one hour. After subcutaneous injections of the same dose, no increase in uterine adrenaline was observed within 10 minutes, in fact the amount present was less than that found in the control uteri. Even 100 μg. doses of adrenaline

TABLE 1. UTERINE ADRENALINE AND NORADRENALINE LEVELS FOLLOWING INJECTIONS OF ADRENALINE

Treatment	Lapsed time (minutes)	No. of rats	Adrenaline μg./g-uterus	Noradrenaline μg./g-uterus
Control (Adrenaline-μg.)		14	0.02 ± 0.00	0.45 ± 0.03
25 ip.†	10	6	*0.84 ± 0.07**	0.35 ± 0.06
25 sc.	10	6	0.01 ± 0.00	0.33 ± 0.07
25 ip.	60	6	*0.20 ± 0.04*	0.31 ± 0.05
10 ip.	60	3	0.04 ± 0.01	0.36 ± 0.03
100 sc.	10	6	0.04 ± 0.01	0.32 ± 0.04
100 sc.	60	6	*0.14 ± 0.02*	*0.90 ± 0.11*

* Results in italics are significantly higher than controls. $P = .01$
† ip. = intraperitoneal injection.
sc. = subcutaneous injection.

injected subcutaneously were not followed by a rise in the adrenaline content of the uterus within 10 minutes but within one hour, it was significantly above the initial level. In some instances, no change was observed in the uterine adrenaline concentration at one hour following a 10 μg. dose of adrenaline given intraperitoneally.

After injections of adrenaline, the noradrenaline concentration in the uterus was not significantly altered except in one experiment in which 100 μg. of adrenaline were given subcutaneously, (Table 1).

DISCUSSION

The data show that the adrenaline content of the uterus is considerably greater after intraperitoneal than after subcutaneous injections of adrenaline. This explains why adrenaline must be given intraperitoneally in order to decrease uterine glycogen and phosphorylase (1, 2) because it is only by this route of administration that adrenaline reaches the uterus in sufficient amounts. The dose of adrenaline (25 μg.) found adequate to permit measurement of both catechol amines in the uterus, was also the minimal dose required to lower uterine glycogen, (also ref. 5). Therefore the critical uterine level of adrenaline necessary to produce this effect within 10 minutes may be assumed to be approximately 0.84 μg. per gm. The metabolic changes are most likely limited to the myometrium (1, 2) and since the measurement of adrenaline is in the whole uterus, it is not possible to designate the critical level of hormone required to produce changes in the myometrium alone.

Uterine muscle apparently has a high threshold for metabolic responses to adrenaline compared with skeletal muscle because 10 μg. doses of the hormone given intraperitoneally were sufficient to affect the glycogen of skeletal muscle (5) but not that of the uterus. Intraperitoneal injection permitted the rapid attainment of the threshold level of adrenaline for the stimulation of these responses. It was noted that one hour after injecting 25 μg. of adrenaline intraperitoneally, the uterine adrenaline fell to 0.20 μg. which is not much greater than the level of 0.14 μg. observed when larger doses of adrenaline were injected subcutaneously. Only in the first instance was uterine metabolism affected. If measurements of uterine adrenaline had not been made within 10 minutes after adrenaline administration misleading interpretations could have resulted.

In the experiments reported here, noradrenaline content of the uterus increased in only one instance, although noradrenaline concentration in both plasma and skeletal muscle has been noted to increase following the injection of adrenaline (3). Large intraperitoneal doses of noradrenaline will also lower glycogen and phosphorylase and it appears likely that the uterine level of noradrenaline would have to be greater than 0.90 μg. per gm. before these metabolic changes are observed.

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THE ENDOCRINE SOCIETY

1959 AWARDS

The selection of the recipients of the awards of The Endocrine Society is made by a Committee appointed by the Council of the Society. These awards and fellowships carry no obligation by the recipient to the Society or to the donors.

Fred Conrad Koch Award

During the past year a substantial legacy has been bequeathed the Society by the late Elizabeth Koch for the purpose of establishing the Fred Conrad Koch Memorial Fund in memory of her late husband, distinguished service professor of physiological chemistry at the University of Chicago and pioneer in the isolation of the androgens. This is to be the highest honor of the Endocrine Society and is to be represented by a medal that is to be known as the Koch Medal of The Endocrine Society. The medal and honorarium of $3,500 is to be given annually to an individual for work of special distinction in endocrinology.

The recipient shall be chosen from nominations presented by members of the Society and is limited to citizens of the United States and Canada. This award will replace the Medal of the Endocrine Society which was established in 1954 and presented to Dr. Carl Moore in 1955, Dr. Frederick L. Hisaw in 1956, Dr. Joseph C. Aub in 1957, Dr. I. L. Chaikoff in 1958 and Dr. Wilbur W. Swingle in 1959. The Endocrine Medal replaced the E. R. Squibb Award which was formerly the highest honor bestowed by the Society. Past recipients of the Squibb Award were Dr. George W. Corner in 1940, Dr. Philip E. Smith in 1941, Dr. Fred C. Koch, 1942, no award was given in 1943, Dr. E. A. Doisy in 1944, Dr. E. C. Kendall in 1945, Dr. Carl O. Hartman in 1946, Drs. Carl F. and Gerty T. Cori in 1947, Dr. Fuller Albright in 1948, Dr. Herbert Evans in 1949, Dr. C. N. H. Long in 1950, Dr. J. B. Collip in 1951, Dr. James H. Means in 1952 and Dr. David Marine in 1953.

The Ciba Award

The Ciba Award, to recognize the meritorious accomplishments of an investigator not more than 35 years of age in the field of clinical or preclinical endocrinology, was established in 1942, but no recipient was selected in 1942 or 1943. In 1944 the Award was presented to Dr. E. B. Astwood, 1945 Dr. Jane A. Russell, 1946 Dr. Martin M. Hoffman, 1947 Dr. Choh Hao Li, 1948 Dr. Carl Heller, 1949 Dr. George Sayers, 1950 Dr. Oscar M. Hechter, 1951 Dr. Albert Segaloff, 1952 Dr. Seymour Lieberman, 1953 Dr. Sidney Roberts and Dr. Clara Szego (Mrs. Roberts), 1954 Dr. Isadore M. Rosenberg, 1955 Dr. Jack Gross, 1956 Dr. Alfred M. Bongiovanni, 1957 Dr. Nicholas S. Halmi, 1958 Dr. Monte Arnold Greer, and 1959 Dr.

Gordon L. Farrell. Prior to 1952 the Award was $1,200. It has now been increased to $2,500.

The Ayerst and the Squibb Fellowships

The Ayerst Fellowship was established in 1947 and the Squibb Fellowship in 1956. They are designed to assist men or women of exceptional promise in furthering their advancement towards a career in endocrinology. Each Fellowship is awarded on alternate years and the stipend, which will not exceed $5,000 may be divided into two Fellowships in varying amounts in accordance with the qualifications of the appointees. Individuals possessing the M.D. or Ph.D. degree, or candidates for either of these degrees, are eligible for appointment.

Applicants must submit the following information:

1. Evidence of scientific ability as attested by studies completed or in progress.
2. Recommendations from individuals familiar with the candidate and his work.
3. A proposed program of study.
4. Acceptance of the individual by the head of the department in which the Fellowship will be held.
5. A statement that he or she will serve full time if awarded a Fellowship. A small amount of time (10 to 15 per cent) may be spent in course work or participation in teaching, the latter purely on a voluntary basis.

The Schering and the Upjohn Scholars of The Endocrine Society

The Council of The Endocrine Society has established a category of Scholars. These grants have been made available through the generosity of the Schering Corporation and the Upjohn Company, and will be awarded to established investigators and teachers in the field of endocrinology who wish to extend their opportunities for work either in this country or abroad.

The awards will not exceed $2,500 annually for each individual and will be granted on the basis of proposals submitted by the applicant. Such applications should include estimated financial needs. The funds may be used for travel, maintenance and other expenses.

Nominations

Nominations for the Fred Conrad Koch Award; the Ciba Award; and the Ayerst and the Squibb Fellowships may be made by any member of The Endocrine Society. They should be submitted on forms which may be obtained from the Office of the Secretary, 1200 North Walker Street, Oklahoma City 3, Oklahoma. Completed nominations should be returned to the Secretary not later than October 15, 1959.

Proposals for appointments as Scholars of The Endocrine Society should be made in writing by the individual, and addressed to the Secretary of the Society. They should be submitted by October 15, 1959.

The Awards Committee will meet in October and notice of awards to successful nominees and applicants will be made not later than December 1st.

CITATIONS FOR THE 1959 AWARDS

The Endocrine Medal

The Fred Conrad Koch Award of the Endocrine Society is the highest award made by the Society and is bestowed on an individual who has made distinguished contributions to endocrinology. This year it is awarded to *Wilbur Willis Swingle*, Henry Fairfield Professor of Biology in Princeton University.

Dr. Swingle received his B.A. and M.A. degrees at the University of Kansas and his Ph.D. degree in biology from Princeton University. He then joined Professor Ross Harrison's department at Yale University, first as instructor then as assistant professor. In 1926 he was appointed Professor and Chairman of the Department of Biology at the University of Iowa. His stay at Iowa, however, was a brief one, for in 1929 he was called to Princeton University as Professor of Biology. He served the University with distinction as the Conklin Professor of Biology until 1956, when having reached the statutory retiring age, he was appointed to the research professorship he now holds.

Throughout his scientific career Dr. Swingle and his numerous colleagues and collaborators have devoted themselves to the study of the function of the endocrine organs. His first paper published in 1917 dealt with the effects of feeding thymus gland to frog larvae and a perusal of his list of publications reveals that, with very few exceptions, the 150 papers appearing under his name have reported the results of his studies on a variety of topics in endocrinology.

His name, however, will always be associated with our knowledge of the adrenal cortical hormones. His first paper on adrenal cortical function was published in 1926 and in this and several subsequent contributions he described the occurrence of hemoconcentration, acidosis, increased blood-urea and hypoglycemia in bilaterally adrenalectomized cats. It is quite evident that these early studies on the profound chemical changes in the blood of animals dying in adrenal insufficiency had a marked influence on his subsequent work and one may surmise turned his thoughts to the possibility of isolating the active principle of the adrenal glands. Others before Swingle had attempted to obtain this active principle. Hartman as well as Stewart and Rogoff had reported that saline extracts of the adrenal prolonged the life of adrenalectomized animals, but none had succeeded in obtaining extracts that restored adrenalectomized animals to full health.

In the last week of 1929 at the meeting of the American Society of Zoologists held at Ames, Iowa, Swingle and his colleague Pfiffner reported they had prepared an extract from the *lipid* fraction of beef adrenals that greatly prolonged the life of adrenalectomized cats. Early in 1930 they were able to state that such extracts would maintain adrenalectomized animals in perfect health for an indefinite period and that withdrawal of the extract was followed by death in typical adrenal insufficiency within a few days. By one of the coincidences that are often associated with a major discovery, Hartman and Brownell in the same journal also reported that a lipid extract was far more effective than water or saline extracts in supporting adrenalectomized animals.

These papers mark a turning point in the history of our knowledge of adrenal cortical function since they demonstrated not only that it was possible to concentrate the active principle of the gland, but what was equally important, that this principle was associated with the lipids of the gland. This clue was soon followed up and by 1937 several laboratories particularly those of Reichstein, Kendall and Wintersteiner had not only isolated active crystalline materials from such lipid extracts, but had identified the active principles as belonging to the steroid group of compounds.

Swingle, however, did not pursue the chemistry of these new principles but instead began an investigation on the nature of their control of bodily functions. No doubt he had been impressed by the profound changes in water and salt metabolism that he and others had observed to follow adrenalectomy, and he and his collaborators soon demonstrated that the administration of the new adrenal extracts rapidly reversed these alterations. In a long series of papers he analyzed these water and electrolyte changes and reached a conclusion that he still holds, that a major function of the adrenal hormones is to facilitate the passage of water and salts between the fluid compartments of the body. Although others have shown that certain adrenal steroids such as desoxycorticosterone and aldosterone probably have a specific effect on electrolyte transfers in the renal tubules, Swingle demonstrated in a most ingenious series of experiments that renal sodium loss and consequent reduced sodium concentration in the extracellular fluid was not in itself entirely responsible for the disordered water and electrolyte metabolism observed in adrenal insufficiency. Indeed in his most recent papers he draws a clear distinction between the renal effects produced by aldosterone and the water shifts between the body compartments produced by cortisone and its allied compounds.

The adrenal gland has always been notorious for the controversies that have raged about its function. It will be recalled that long before much attention was paid to the cortical portion of the gland, the nature of the medullary secretion had aroused a heated debate. Soon after the introduction of potent adrenal extracts controversy also arose as to the prepo-

tent function of the adrenal cortex. Many of you who are present this evening will remember the often sharp differences of opinion between Dr. Swingle and his colleagues on the one hand, and Dr. Britton and his collaborators as to the primary effect of these hormones. As has been stated, Swingle from his studies emphasized the importance of the control of the water and salt metabolism, while Britton from his work inclined to the view that it was the evident disturbances in carbohydrate metabolism of adrenalectomized animals that were to be considered as indication of the major site of action of these hormones.

Although we are still far from an understanding as to the manner by which a single hormone can influence both electrolyte and water transfers as well as carbohydrate and protein metabolism, nonetheless we now know that the observations of both laboratories were correct and, as is often the case in scientific controversy, it was the interpretation of the evidence rather than the evidence itself that was the cause of the differing opinions.

The measure of a scientist is to be found not only in his own contributions to knowledge but also in the number and distinction of his pupils. Dr. Swingle has fulfilled both these criteria to an admirable degree. Up to the present time no less than thirty-one individuals have completed the work for the Ph.D. degree in his laboratory. Many of these hold professorial positions, other positions of importance in the research laboratories of government or industry. The record of their achievement must be a great source of satisfaction to their old chief and their continued loyalty and admiration of him must be as proud a distinction as that the Endocrine Society confers on him this evening.

Dr. Swingle, the Endocrine Society is happy to present to you the first Fred Conrad Koch Award. It does so in recognition of your great contributions to endocrinology not only as an investigator but also as a great teacher who has been the inspiration of so many students who, in their turn, have broadened and enriched the subject that has been your life work.

Ciba Award

The Ciba Award for 1959 has been made to Gordon L. Farrell for outstanding contributions in the role of ACTH and adrenal cortical hormone secretions.

Dr. Farrell was born on April 16, 1925. He attended Carroll College in Helena, Montana, 1940–43 and received the M.D. in 1947 at St. Louis University. He then held the following positions: Intern, U.S.P.H.S. Hospital, New Orleans, 1947–48; Assistant Surgeon, U.S.P.H.S. 1948–1951; Instructor, Department of Pharmacology, University of Utah, 1951–52; Research Fellow, Department of Physiology, Western Reserve University, 1952–54; Instructor, 1954–56, and Assistant Professor, 1956–.

Dr. Farrell was the first to demonstrate that desoxycorticosterone and substance S are secreted by the adrenal cortex. These studies were followed

by the isolation and characterization of aldosterone from the adrenal venous blood of the dog.

The application of quantitative procedures to the detection of ACTH in blood demonstrated that epinephrine increased the level in rat blood to a detectable amount and long term administration of cortisone inhibited the synthesis of ACTH at the pituitary level. The extension of these studies to hypophysectomized animals yielded an understanding of the time relationship between ACTH release and steroid synthesis in the adrenal cortex. A comparison of the ability of various ACTH preparations to stimulate adrenal cortical secretion indicated that separate factors were present for the secretion of aldosterone and cortisol.

The physiological factors which regulate aldosterone secretion have been studied intensively by Dr. Farrell during the past few years. Investigations of the effects of hypophysectomy, central nervous system ablations and brain stem lesions led to the discovery that areas important in aldosterone regulation exist in the posterior diencephalon and anterior midbrain. This property has been demonstrated to be due to a new hormone which can be extracted from the diencephalon or the pineal complex. Dr. Farrell has given the hormone the conditional name of "glomerulotropin."

Dr. Farrell's more recent studies have been concerned with the influence of changes in blood volume, of heart failure and of alteration in electrolyte intake on the rate of aldosterone secretion.

Dr. Farrell now is extending his basic studies and applying this new knowledge to studies on patients with aldosterone secreting tumors and patients with hypertension.

Upjohn Scholar

The Upjohn Scholar for 1959 is Harold L. Mason. Dr. Mason has had a distinguished career in steroid endocrinology. He collaborated with Dr. E. C. Kendall in the pioneer studies on the isolation, characterization and synthesis of the adrenal cortical steroids.

Dr. Mason then turned his acquired skill in steroid chemistry to the isolation from the urine of patients of many hitherto unrecognized metabolites of not only the adrenal cortical steroids but also of the androgens. More recently he tackled the question of the active material in the amorphous fraction and announced the successful isolation of aldosterone only shortly after that of the European team of investigators.

Dr. Mason was born in Comptom, California, April 17, 1901. He earned the B.A. degree at the University of Southern California, 1923, A.M., 1924, and the Ph.D. (Organic Chemistry), 1927, University of Chicago. He was an instructor in chemistry at Northwestern University, 1927–28. In 1928 Dr. Mason was appointed professor of physiological chemistry at the Mayo Foundation and became head of the department after Dr. Kendall's retirement.

The scholarship will be used by Dr. Mason to do some collaborative work at the Worcester Foundation for Experimental Biology and the National Institutes of Health. The time spent at these two institutions are certain to benefit not only Dr. Mason but probably bring even greater benefit to the laboratories.

Schering Scholar Award

Henry Hubert Turner was born in Harrisburg, Illinois, in 1892. He was educated in Harrisburg, at St. Louis University, and received an M.D. degree from the University of Louisville School of Medicine. He took postgraduate training at the University of Vienna and in Queen's Square, London.

Henry, affectionately known to his close intimates as Jack, has been prominent in American Medicine for many years. He became Associate Professor of Medicine at the University of Oklahoma School of Medicine in 1939, and in 1949 was made Clinical Professor of Medicine. He is widely known as a lecturer especially in endocrinology before medical societies in various parts of the country and at many postgraduate courses of the American College of Physicians and the postgraduate assemblies of the Endocrine Society. He is a Diplomate of the American Board of Internal Medicine and a member of many medical societies.

He is perhaps most widely known for his paper entitled "A Syndrome of Infantilism, Congenital Webbed Neck, and Cubitus Valgus" published in *Endocrinology* in 1938. This is a subject on which he has lectured and written since and has modestly called ovarian agenesis but which most others know as "Turner's Syndrome." He has been an active member of the Endocrine Society for many years and has been its hard-working Secretary since 1941. His present interest is a follow-up study of testicular histology and physiology especially in relationship to former treatment with chorionic gonadotropin. The award of the Schering Scholarship will make it possible for him to visit various endocrine centers in this country and abroad in which he can gather further information in the line of his present investigation.

Ayerst Fellowship

The recipient of the Ayerst Fellowship for 1959 is Henry Angus Boright. Dr. Boright was born in Montreal, Canada, on March 15, 1929, and obtained his collegiate training at McGill University. He was granted the B.Sc. degree in 1951 and the M.D. in 1955. From 1956–1957 he was an interne in medicine at the Royal Victoria Hospital. The following year, 1957–1958, was occupied as a research fellow in the Endocrine-Metabolic Unit at the Royal Victoria Hospital, Montreal, studying the relationship of serum mucopolysaccharides to the development of renal disease in diabetes. This project provided the material for a thesis towards the M.Sc.

degree and also two publications. During the past year Dr. Boright has been an assistant resident in the Department of Medicine at the Royal Victoria Hospital.

Dr. Boright now will leave the cold of Montreal to spend a year in the warmth of North Carolina where he will work in the laboratories of Dr. Frank Engel at Duke University. The studies will be concerned with the relationship of growth hormone to fat metabolism in experimental animals.

PROGRAM OF THE FIRST INTERNATIONAL CONGRESS OF ENDOCRINOLOGY

The program of this meeting, which will be held in Copenhagen from July 18 through 23, 1960, will consist of ten Symposia and a Round Table Discussion and of groups of shorter (ten minute) communicated papers. Speakers at the Symposia and the Round Table Discussion are invited by the Congress. Communicated papers may be submitted by investigators in the field of endocrinology, and titles and abstracts are invited. Forms for registration in the Congress, along with forms for the submission of abstracts of short communications, may be obtained by writing to the Congress Secretariat: Dr. Svend G. Johnsen, Hormone Department, Statens Seruminstitut, Copenhagen S, Denmark. The final date for the submission of such abstracts is December 31, 1959. The official languages of the Congress are English, French, German, and Spanish. A volume containing advance abstracts of symposium contributions and the short communications (in one of the official languages and translated into Interlingua) will be distributed to members on registration at the Technical University of Denmark in Copenhagen. Titles and abstracts of communicated papers should not be sent to the Program Committee, but the Program Committee will review and arrange for the presentation of such papers in appropriate Congress sessions.

GREGORY PINCUS, *Chairman*
Subcommittee on Program
First International Congress
of Endocrinology

THE AMERICAN GOITER ASSOCIATION

The Fourth International Goiter Conference will be held July 5–9, 1960, in London, England, under the auspices of the London Thyroid Club and the American Goiter Association. The American Goiter Association plans to make available to worthy candidates a limited number of travel grants for participants of this meeting.

Application blanks are available from John C. McClintock, M.D., 149½ Washington Avenue, Albany 10, New York, U.S.A.

Applications will be received until January 1, 1960.

AMERICAN UROLOGICAL ASSOCIATION, INC.

Urology Award—The American Urological Association offers an annual award of $1000 (first prize of $500, second prize $300 and third prize $200) for essays on the result of some clinical or laboratory research in Urology. Competition is limited to Urologists who have been graduated not more than ten years, and to hospital interns and residents doing research work in Urology.

The first prize essay will appear on the program of the forthcoming meeting of the American Urological Association, to be held at the Palmer House, Chicago, Illinois, May 16–19, 1960.

For full particulars write the Executive Secretary, William P. Didusch, 1120 North Charles Street, Baltimore, Maryland. Essays must be in his hands before December 1, 1959.

MEMOIRS OF THE SOCIETY FOR ENDOCRINOLOGY

NO. 6. IMPLANTATION OF OVA

The attention of members of the Endocrine Society is drawn to the publication of this Memoir which reports the proceedings of a Symposium held in London to survey nidation of the mammalian ovum. The various factors—hormonal, biochemical and pharmacological—concerned are discussed in the light of recent research, most of it previously unpublished.

The volume consists of about 100 pages of text and is extensively illustrated with text-figures and plates.

Members may obtain a single copy at $4.00 by sending their check to the Treasurer. Additional copies may be obtained at a price of $6.00 through booksellers or from the publishers, Cambridge University Press, 32 East 57th Street, New York 22, N.Y.

ERRATUM

The names of Kenneth Savard and Ralph I. Dorfman, co-authors of the paper entitled "Conversion of C^{14}-Testosterone to C^{14}-Estrogenic Steroids by Endocrine Tissues" by Billy Bagget *et al.* (ENDOCRINOLOGY 64:600. 1959) were inadvertently omitted from:

a) The table of contents of the April 1959 issue of ENDOCRINOLOGY
b) The contents of Volume 64 of ENDOCRINOLOGY (which appeared in the June 1959 issue)
c) The author index for Volume 64
d) The entry for Bagget, Billy, in the author index for Volume 64

The correct citation should read: Bagget, Billy, Lewis L. Engel, Luzmaria Balderas, Gertrude Lanman, Kenneth Savard and Ralph I. Dorfman: Conversion of C^{14}-Testosterone to C^{14}-Estrogenic Steroids by Endocrine Tissues. ENDOCRINOLOGY 64:600. 1959